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# The Effects of Novel Serms on Endothelial and Epithelial Tumor Cell Estrogen Receptor Activation

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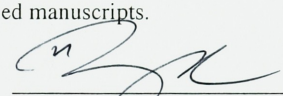

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# THE EFFECTS OF NOVEL SERMS ON ENDOTHELIAL AND EPITHELIAL TUMOR CELL ESTROGEN RECEPTOR ACTIVATION

**A Thesis Submitted to the**
**Yale University School of Medicine**
**In Partial Fulfillment of the Requirements for the**
**Degree of Doctor of Medicine**

**by**
**PAVLOS ZACHARIAS KAIMAKLIOTIS**
**2006**

**Abstract**

Premenopausal women are relatively protected against the development of coronary heart disease, when compared with age-matched men. This difference dissolves after menopause and is accepted to be hormonally mediated. Despite recent clinical controversies in the use of hormone replacement therapy in postmenopausal women, there have been numerous *in vitro* and *in vivo* studies that demonstrate favorable effects of estrogen on the endothelium. The Bender laboratory has shown that 17β-estradiol ($E_2$) rapidly induces nitric oxide (NO) release from human endothelial cells (EC) *in vitro*. This occurs through a c-Src/phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathway, in a rapid fashion in the absence of modulated gene expression. In addressing how these membrane growth factor receptor-type responses to a steroid hormone could occur, the Bender laboratory also demonstrated that EC have more than one form for ERα. The classical ERα, a 66kDa protein, is predominantly cytosolic and nuclear, and comprises a minority of the ERs in most EC. ER46, a 46kDa protein, is a product of an alternative transcript splice form, and represents a majority of the ERs in most EC. ER46 has a predilection for membrane targeting, and more efficiently transduces the favorable EC activation responses to $E_2$ described above. Given the controversy surrounding the effects of hormone replacement therapy in postmenopausal women, the design and use of selective estrogen receptor modulators (SERMs) has recently gained great attention and hope that some of these compounds will have beneficial effects on vascular and bone cells, without the detrimental side/toxic effects.

The hypothesis is that, of a selected panel of potential SERMs, there will be a hierarchy of efficacy and toxicity, with regard to rapid induction of endothelial nitric

oxide synthase (eNOS) activation through EC membrane ER46, and to proliferative responses of breast cancer cells, respectively. A compound that is found to be highly efficient at inducing eNOS activation, and minimally pro-proliferative, may be a tremendously useful prophylactic and threapeutic agent in humans.

## Acknowledgements

I would like express my gratitude to my faculty advisor and mentor Dr. Jeffrey Bender for allowing me the opportunity to undertake this project. I would also like to thank Dr. Bender for his patience, encouragement, and invaluable advice on my career decisions during my four years at Yale University School of Medicine. His enthusiasm and love for medicine and research have been an inspiration as I start my career in medicine. I would like to acknowledge and thank Lei Li for helping me get started in the lab. I would also like to thank Lynn O'Donnell and Eleni Skokos for their help in the lab along the way. Finally, I would like to thank my parents, Zack and Myroulla for their endless sacrifices and support the past four years.

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## 1.0 Introduction

As early as the 1950s, animal studies have shown that exogenous estrogen could inhibit coronary atherosclerosis, when it was observed that estrogen inhibited coronary atherosclerosis in cholesterol fed chicks (1). Studies by Harder and Coulson in the late 1970s (2) showed that estrogen binds to coronary artery smooth muscle cells in a canine model. In the early 1990's, Williams et al. (3) performed a number of studies of estrogen's effects in female cynomolgus monkeys. The initial studies performed in ovariectomized females demonstrated that long-term estrogen treatment improved vasomotor function and reduced atherosclerosis. It was not clear, however, whether this was a direct vascular effect or an effect mediated via changes in circulating lipoproteins. Williams et al. (4) later attempted to determine the effects of short term estrogen administration on the vascular responses of atherosclerotic arteries in ovariectomized cynomolgus monkeys. The vascular responses of coronary arteries to the endothelium-mediated vasodilator acetylcholine were reversed from abnormal vasoconstriction to vasodilation within 20 minutes after an infusion of ethinyl estradiol. This reversal was not accompanied by any changes in plasma cholesterol levels, demonstrating a direct effect of estrogen on the vascular endothelium.

Even though the cardioprotective effects of estrogen in women are believed to be mediated, in part, by its effects on lipoprotein levels, the studies by Williams et al (4) support the importance of direct effects of estrogen on the vasculature. Since then, studies have helped to further elucidate the cardiovascular effects of hormone replacement therapy (HRT) and the importance of timing of therapy initiation.

## 2.0 Effects of Estrogen on Vascular Endothelium Function

Vascular endothelial cells play an important role in modulating the tone of the underlying vascular smooth muscle by the synthesis and release of vasodilator factors such as NO, prostacyclin, endothelium derived hyperpolarizing factor, and vasoconstrictor factors such as $PGH_2$, endothelin, and oxygen derived free radicals, as described in Figure 1 below (5).

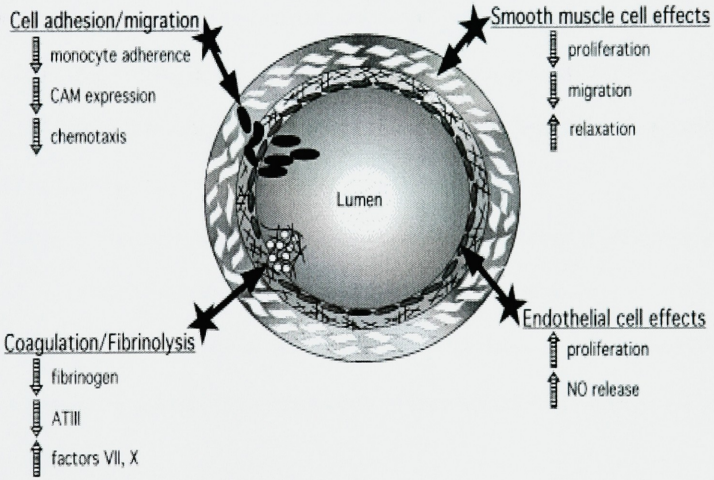



**Figure 1 – Direct Effects of Estrogen on Blood Vessels** (From Haynes et al., Rapid vascular cell responses to estrogen and membrane receptors, *Vascul. Pharmacol.* **38** (2002), pp. 99–108)

## 2.1 Up-regulation of Endothelial Nitric Oxide (eNO) Production

Early studies indicated the possible influence of sex steroid hormones on eNO synthesis by demonstrating gender differences in endothelium-dependent modulation of vascular tone (6-8). Studies on isolated rat aorta showed that the vascular endothelium of female WKY rats is capable of producing larger amounts of NO under both basal and stimulated conditions than that of male WKY rats (9). Further studies investigating the effect of ovariectomy and/or estrogen treatment on endothelium-dependent vascular responses supported the hypothesis that the greater production of eNO in females is due to the effect of the female sex steroid hormone (17β-estradiol) (8,10,11). Gisclard et al. (1-6) described increased endothelium dependent relaxation in isolated femoral arteries of rabbits after 4 days of treatment with 17β-estradiol. Hayashi et al. 1-4 showed that basal release of NO from female rabbit aorta was greater than that from male or ovariectomized female rabbit aortas.

Clinical investigations have also demonstrated attenuation of abnormal vasomotor responses by administration of ethinyl-estradiol (12) or physiological doses of 17β-estradiol (13). A two year follow up study reported increases in circulating NO levels in postmenopausal women on HRT compared to women not taking estrogen (13).

## 2.2 Mechanisms Involved in Estrogen Mediated Increased eNO Production

### 2.2.1 Genomic Effects

The longer-term effects of estrogen are produced, at least in part, by changes in vascular cell gene and protein expression. These effects are mediated by ligand activated transcription factors, estrogen receptors ER-α and ER-β, which are members of the

nuclear hormone receptor superfamily. Although both ER-α and ER-β are expressed in endothelial (14) and vascular smooth muscle cells (15,16), some data show that ER-β expression predominates in endothelial cells, whereas ER-α expression is greater in smooth muscle cells. When estrogen binds to its receptors (see Figure 2), the estrogen-ER complexes undergo homodimerization or heterodimerization, (17) after which they are able to bind specific DNA sequences in the control regions of their target genes and upregulate transcription of these genes. The estrogen-ER complex interacts with several

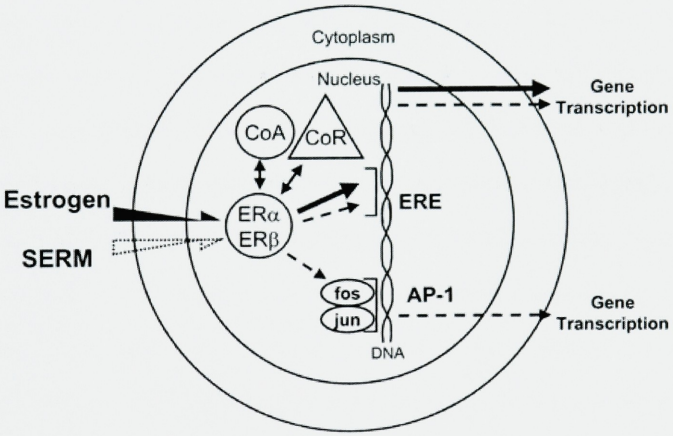



**Figure 2 - Mechanism of Estrogen-Receptor Activation of Gene Expression.** The signal transduction pathways available to estrogen or a selective estrogen receptor modulator (SERM) to initiate gene transcription. Estrogen binds to either estrogen receptor ER-α or ER-β and subsequently binds coactivator (CoA) molecules required to form a transcription complex at an estrogen response element (ERE) located in the promoter region of an estrogen-responsive gene. The antiestrogenic action of a SERM results from the inappropriate folding of an ER-α or ER-β complex that either cannot recruit CoA molecules or instead recruits corepressor (CoR) molecules. This programmed change in conformation produces antiestrogen action at specific sites like the breast but estrogen-like effects in the uterus if an excess of CoA molecules is present. These events modulate gene transcription through EREs. SERM–ER complexes may also initiate gene transcription to produce an estrogen-like effect, by forming a protein–protein interaction at fos/jun that activates activating protein (AP)-1 sites. In addition, SERMs may produce nongenomic effects and alter tissue biochemistry without interacting with ERs (From Jordan et al., Journal of the National Cancer Institute, 2001, Vol. 93, N0 19, 1449-1457).

proteins that are recruited to the receptor after ligand binding and facilitate transcriptional activation. These ER associated proteins have the ability to further recruit transcription proteins, and in some cases have enzymatic functions that facilitate target gene expression (18). Control of gene expression by the estrogen-ER complexes, thus, involves a series of specific molecular interactions among estrogens, ERs, ER-associated proteins, and the precise control regions for the estrogen target genes present in a given cell.

ERs in vascular cells alter the expression of a number of such specific target genes (see Table 1). Important examples of such genes in the cardiovascular system include: prostacyclin cyclooxygenase, prostacyclin synthase, endothelial nitric oxide synthase, inducible nitric oxide synthase, endothelin-1, collagen, matrix metalloproteinase, vascular cell adhesion molecules, vascular endothelial growth factor, elastin, and progesterone receptor (16). The protein products of these genes, in turn, are thought to mediate specific genomic effects of estrogen on vascular tissue in an autocrine and/or paracrine manner (19). Even though ERs are by all evidence to date identical, regardless of where they are expressed, it is thought that the receptor associated proteins that are recruited after ligand binding provide a first level of specificity leading to cell specific events. These coactivator/repressor proteins may be expressed in different ratios in various cell types, and may also assemble in unique combinations that allow for a certain degree of specificity. Additional cell specific ER associated coactivator or coregulatory proteins may also exist (20). These tissue-specific or cell-specific ER-associated coactivator proteins represent attractive targets for drug discovery.

A second level of target tissue specificity is likely provided by cis-acting elements specific to the genes that are regulated by estrogen in a given cell. For example, the gene for eNOS is upregulated by estrogen in vascular cells but not in bone or reproductive tissues (19). This means that the genes activated by estrogen in vascular cells are different from those activated in bone cells, and the regulatory regions of these genes likely dictate some of the specificity of a gene in a given cellular environment.

**TABLE 1.** ESTROGEN-REGULATED GENES OF POTENTIAL IMPORTANCE IN VASCULAR PHYSIOLOGY AND DISEASE.

| GENE PRODUCT | PHYSIOLOGIC OR PATHOPHYSIOLOGIC ROLE* |
|---|---|
| **Candidate estrogen-regulated genes (vascular cells)** | |
| Prostacyclin synthase | Vasodilatation |
| Endothelial nitric oxide synthase | Vasodilatation |
| Inducible nitric oxide synthase | Vasodilatation in response to vascular injury |
| Endothelin-1 | Vasoconstriction |
| Collagen | Vascular-matrix formation |
| Matrix metalloproteinase 2[23] | Vascular-matrix remodeling |
| E-selectin | Cell adhesion |
| Vascular-cell adhesion molecule | Cell adhesion |
| Vascular endothelial growth factor | Angiogenesis and endothelial-cell proliferation |
| **Candidate estrogen-regulated genes (nonvascular cells)** | |
| Growth- and development-related genes | |
| Transforming growth factor $\beta_1$[24] | Wound healing |
| Epidermal growth factor receptor | Cell growth in response to vascular injury |
| Platelet-derived growth factor[25] | Cell growth in response to vascular injury |
| flt-4 tyrosine kinase | Angiogenesis and endothelial-cell proliferation |
| Coagulation- and fibrinolysis-related genes | |
| Tissue factor[26] | Hemostasis in response to thrombosis |
| Fibrinogen | Hemostasis in response to thrombosis |
| Protein S | Hemostasis in response to thrombosis |
| Coagulation factor VII | Hemostasis in response to thrombosis |
| Coagulation factor XII[27] | Hemostasis in response to thrombosis |
| Plasminogen-activator inhibitor 1 | Hemostasis in response to thrombosis |
| Tissue plasminogen activator[28] | Fibrinolysis |
| Antithrombin III | Anticoagulation |
| Signaling-related and miscellaneous genes | |
| Estrogen receptor $\alpha$ | Hormonal regulation and gene expression |
| Estrogen receptor $\beta$ | Hormonal regulation and gene expression |
| Monocyte chemotactic protein 1[29] | Monocyte recruitment and atherosclerosis |
| $I_{SK}$ and HK2 (cardiac potassium channels)[30] | Cardiac conduction |
| Connexin 43 | Cardiac conduction |
| Leptin[31] | Fat metabolism and obesity |
| Apolipoproteins A, B, D, and E and Lp(a) | Lipid metabolism and atherosclerosis |
| Angiotensin-converting enzyme | Vasoconstriction |
| Angiotensin II receptor, type 1 | Vasoconstriction |

**Table 1- Estrogen-Regulated Genes of Potential Importance in Vascular Physiology and Disease.** (From Mendelsohn et al., The Protective Effects of Estrogen on the Cardiovascular System N Engl J Med 1999 **340**: 1801-1811)

Approximately a third of the protective cardiovascular effects of estrogen can be attributed to modification of known risk factors by estrogen, such as modulation of the levels or activities of lipoproteins and apoproteins and of coagulation and fibrinolytic proteins. These effects are primarily those of estrogen on ER-α in hepatic cells (19).

### 2.2.2 Nongenomic Effects

Estrogen can increase the bioavailability of eNO in a variety of models (19,20). Administered directly into the coronary artery of a nonhuman primate or a human, estradiol causes rapid vasodilation by a activating eNO production (19).

The molecular mechanisms by which estrogen activates eNOS is currently an area of active interest. In 1997 Lantin-Hermoso et al. (22) and Caulin-Glaser et al. (21) reported that relatively specific antagonists of the ERs could inhibit nitric oxide release form cultured endothelial cells exposed to physiologic concentrations of estrogen. Lantin-Hermoso et al. (22) demonstrated that eNOS activity increased in a dose dependent manner within 5 minutes after ovine pulmonary artery endothelial cells were exposed to 17β-estradiol. In these studies, removal of ionic calcium completely inhibited eNOS activity stimulated by 17β-estradiol. This supported the hypothesis that 17β-estradiol acutely stimulates eNOS by increasing calcium influx. The ER antagonist ICI-182780 fully inhibited eNOS activity stimulated by 17β-estradiol in these studies. Caulin-Glaser et al (21) demonstrated a rapid increase in nitric oxide release from endothelial cells form the female human umbilical vein in response to 17β-estradiol. Similarly, they found that the ER antagonist ICI 164,384 inhibited this increase in nitric oxide release.

In 1999, Chen et al. (23) showed that ER-α can mediate the short term effects of estrogen on eNOS activity. 17β-estradiol causes activation of eNOS within 5 minutes. This activation was specific to 17β-estradiol, required the ER-α hormone binding domain, and was blocked by ER antagonists. The acute response of eNOS to 17β-estradiol could be reconstituted in COS cells cotransfected with ER-α and eNOS, but not in those transfected with eNOS alone. Additionally, 17β-estradiol caused the rapid, ER-dependent activation of the mitogen-acivated protein (MAP) kinase, and the MAP kinase inhibitor PD-98059 blocked the ability of 17β-estradiol to activate eNOS in intact endothelial cells. These findings suggested that the rapid activation of eNOS is mediated by ER-α functioning in a nongenomic way. The data from this study also suggest that the MAP kinase pathway might be recruited by activation of ER and be involved in eNOS activation.

In 2000 Haynes et al. (24) provided evidence for the involvement of a second signal transduction pathway in eNOS activation, and demonstrated that PI 3-kinase is capable of activating Akt (protein B kinase, a serine/threonine kinase down-stream of PI 3-kinase). Akt, in turn, directly phosphorylated and activated eNOS. Simonicini et al. (25) showed that human recombinant ER-α binds in a ligand-dependent manner to the p85a regulatory subunit of PI 3-kinase. Stimulation with estrogen increased ER-α associated PI 3-kinase activity, leading to activation of Akt and eNOS. Chambliss et al. (26) also reported in 2000 that the ER-eNOS signaling system is localized to endothelial cell caveolae (see Figure 3).

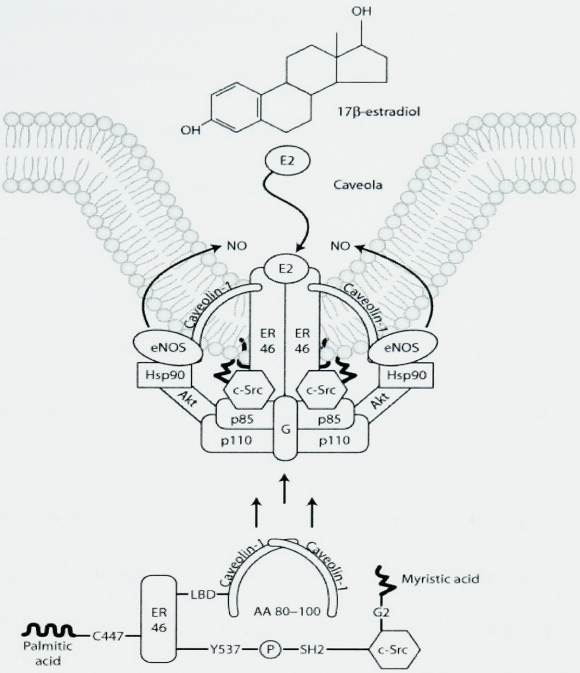



**Figure 3 – Signal Transduction Pathway in eNOS Activation.** (From Kim et al., Rapid, Estrogen Receptor–Mediated Signaling: Why Is the Endothelium So Special? Sci. STKE 2005 (**288**), pe28.)

## 3.0 The Effect of Increased Endothelial Nitric Oxide Production in the Anti-Atherosclerotic and Antihypertensive Effects of 17β-estradiol.

### 3.1 Atherosclerosis

Endothelial cell injury or endothelial dysfunction is considered to play an important role in the development of atherosclerotic lesions (27,28). One of the manifestations of endothelial cell dysfunction is an impairment or reduction of NO production. In 1990, Williams et al. (29) suggested that the vasodilatory effect of estrogen may be mediated by increased NO production by showing enhanced

acetylcholine-induced, endothelium-dependent relaxation of coronary arteries following 17β-estradiol treatment of cholesterol-fed cynomolgus monkeys. Chronic inhibition of NO formation accelerated atherosclerotic progression in hypercholesterolemic rabbits (30,31) and apolipoprtotein E deficient mice (32), further supporting the role of increased eNO production in mediating the anti-atherosclerotic effect of 17β-estradiol.

In 1999 Nascimento et al. (33) investigated the role of NO in mediating the protective effect of 17β-estradiol on atherosclerotic lesion development in ovariectomized, hypercholesterolemic rabbits. Their results showed diminished aortic lesion formation in the estrogen-treated animals accompanied by enhanced endothelium-dependent vasorelaxation of isolated aortic rings, suggesting that the anti-atherosclerotic effect of 17β-estradiol could be mediated, at least in part, by increased NO formation in the aortic endothelium. In the same study chronic, systemic administration of L-NAME to 17β-estradiol-treated hypercholesterolemic rabbits significantly attenuated the anti-atherosclerotic effects of estrogen. Similarly, Holm et al. (34) reported that long-term inhibition of NO synthesis by L-NAME significantly reduced the anti-atherogenic effect of 17β-estradiol and levormeloxifene, a partial estrogen receptor agonist. Levels of plasma cholesterol were fixed, thus demonstrating that the beneficial effect of estrogens in this model is dependent on eNO production and independent of plasma cholesterol lowering. The results of these experiments demonstrate a strong correlation between elevated NO production and anti-atherosclerotic effects of estrogen in this model. However, other factors may also be involved that are able to mediate estrogen-induced protection against atherosclerotic plaque development, including inhibition of VCAM-1 up-regulation and monocyte extravasation (34).

### 3.2 Hypertension

It has been shown that women of reproductive age are less prone to hypertension and hypertension-related diseases than men or postmenopausal women (34). Studies by Winber et al. in 1995 (35) using 24-h ambulatory blood pressure monitoring showed that blood pressure is higher in men than in women at similar ages. These gender-associated differences in blood pressure observed in humans have also been documented in hypertensive animal models. It has been shown that male spontaneously hypertensive rats (SHR) (36), male Dahl salt-sensitive (DS) rats (37) and male deoxycorticosterone-salt hypertensive rats (38) have higher blood pressure than their females counterparts. The important role of ovarian steroid hormones in blood pressure regulation is further supported by the observation that blood pressure increases after menopause in individuals (39). Reducing estrogen levels by ovariectomy aggravates hypertension in DS rats (40), and estrogen replacement has been shown to decrease blood pressure in postmenopausal women (41).

One possible mechanism that may explain the antihypertensive effect of 17β-estradiol is augmentation of endothelial NO production. The aorta of female SHR produces more NO than that of male SHR, and 17β-Estradiol increases eNO mediated vasodilation in female hypertensive rats (42). These studies in rats have been confirmed by clinical investigations, demonstrating attenuation of abnormal vasomotor responses by administration of ethinyl-estradiol (44) or by physiological doses of 17β-estradiol (45).

## 4.0 The Effects of Estrogen on Angiogenesis

### 4.1 Angiogenesis Related to Ovulation and Pregnancy

The majority of angiogenesis seen in mammals occurs during embryogenesis. Adult animals demonstrate a down-regulation of angiogenic responses compared to developing embryos under nonpathological conditions. The exceptions are found in females where during the maturation of the ovarian follicle, the priming of the endometrium for implantation and development of the embryo, adult angiogenesis is essential (46-50). During the menstrual cycle, increased estrogen production and angiogenesis are temporally related (51). The preovulatory surge in circulating 17β-estradiol plasma level is accompanied by a high degree of angiogenic activity (52). In the rat ovary, rapidly dividing endothelial cells produce vessels to support the developing corpus luteum (53). Rapid angiogenesis just after the estrogen surge produces the coiled arteries of the endometrium (54). On the other hand, withdrawal of estrogen in cattle induces rapid vessels regression (47). These studies suggest that ovarian steroid hormones, such as 17β-estradiol, may stimulate angiogenesis.

### 4.2 17β-estradiol Promotes Angiogenesis

Injury to the vessel wall or disruption of the basement membrane that surrounds the capillaries leads to activation of endothelial cells. The cells secrete extracellular matrix proteases, permitting them to migrate into the stromal space and to attach to other matrix molecules. Proliferation yields a sufficient mass of cells to permit organization

into new tubular structures. With secretion and remodeling of a new basement membrane, and attachment of pericytes, a mature capillary is formed (55).

In 1995 Morales et al. (56) showed that estradiol promotes new vessel formation both *in vitro* and *in vivo*. When endothelial cells are plated on a layer of basement membrane matrix (Matrigel), the cells attach, migrate and form tube-like structures with a lumen (57). This morphological differentiation mimics some of the steps involved in angiogenesis *in situ*. This observation suggested that angiogenesis *in vivo* might also be controlled by estradiol. Morales and colleagues employed a model in which βFGF, mixed into the basement membrane extract Matrigel, is injected subcutaneously in mice. Matrigel is a liquid at 4 °C but polymerizes at 37 °C to form scaffolding that is subject to vascular invasion and can be removed subsequently for analysis. Without βFGF, little endothelial cell invasion is observed after 7 days. However, when βFGF is added to the Matrigel, there is marked invasion of cellular elements that are almost entirely von Willebrand factor-positive. Organized vessels are present and contain erythrocytes. If mice are subjected to ovariectomy 2 weeks before implanting the Matrigel plug, there is a marked reduction in cellular invasion to the plug, which is restored by estrogen treatment (56).

Estrogen has also been suggested to be proangiogenic in ischemic tissues since intramuscular injection of the ovarian steroid hormone significantly increased collateral vessel formation in a rabbit hind-limb ischemia model (58).

### 4.3 Potential Mechanisms Involved in the Proangiogenic Effect of Estrogen

The mechanisms involved in the proangiogenic effects of estrogen are multifactorial and include up-regulation of expression of both VEGF and its receptors (59-63), βFGF (64), augmentation of NO synthesis (65), induction of expression of vascular adhesion molecules (66) and integrins (67,68), and inhibiting endothelial cell apoptosis (69,70). The surface expression of integrins β1, α5 and α6 on endothelial cells is regulated by estradiol. Estradiol has a direct effect and does not require the presence of inflammatory cytokines. Since integrins have an important role in mediating endothelial cell attachment, migration, growth and differentiation (67), the increase in integrin expression and function may be an important mechanism by which estrogen regulates angiogenesis.

Estrogen stimulates VEGF production in rats (63), monkeys and humans (59). These studies indicate that at least one mechanism by which estrogen may stimulate angiogenesis is through up-regulation of expression of VEGF and VEGF receptors. However, the angiogenic effects of estrogen are not limited to the tissues important for reproductive biology, nor are they mediated only by VEGF. In 1991, Schecter and Weiner (68) reported that estrogen induces βFGF production by gonadotropic cells in the periphery of the pituitary in Fischer 344 rats. These peripheral sites were adjacent to areas of increased capillary formation required to support ongoing glandular hypertrophy.

## 5.0 Estrogen and Breast Cancer

Estrogen, in addition to other hormones, has an essential role in the development of the female sex organs, the secondary sex characteristics, the regulation of the

menstrual cycle and reproduction. Therefore, it has been proposed that the effects of many well-established reproductive risk factors for breast cancer are mediated by hormonal mechanisms, for the most part involving estrogens (71).

One of the major concerns about the use of long-term HRT is the link between estrogen and the development of breast cancer. Epidemiological and experimental evidence implicates estrogens in the etiology of breast cancer, and most established risk factors for breast cancer in humans are thought to influence risk through hormone-related pathways (71). Increased concentrations of endogenous estrogens are strongly associated with increased risk for breast cancer in postmenopausal women (72), and trials have shown that the anti-estrogens tamoxifen and raloxifene reduce the incidence of breast cancer (73). In an analysis of 51 studies, the relative risk of breast cancer was 1.35 (95% confidence interval = 1.21 to 1.49) for women who currently or recently (i.e., within the past 4 years) used HRT for 5 years or more (74). In the Breast Cancer Detection and Demonstration Project, the risk of breast cancer increased by 8% per year of HRT (75). However, the effects of estrogen alone do not fully account for the relationships observed between breast cancer and hormone-related risk factors. Other hormones, such as progesterone (71), prolactin (76), and testosterone (77), may also be important.

### 5.1 Proliferative Effects of Estrogen on Breast Tissue

Estrogen has been shown to have a marked proliferative effect on breast epithelial tissue in model systems (78). Both endogenous and exogenous estrogens stimulate breast epithelial cell mitosis, increasing the number of cell divisions and thus the opportunity for random genetic errors (71,79). Estrogen concentrations may be important at all stages in

the development of breast neoplasms because the hormonal stimulus to cell division continues all along the progression pathway (80). The proliferative effects of estrogens are brought about on entering target cells, where they bind with a receptor protein, which then binds to hormone response elements on the nuclear DNA, activating or suppressing specific sequences in the regulatory regions of genes responsive to estrogen that control cell growth and differentiation (81).

It has also been suggested that estrogens might have an important influence on risk for developing breast cancer through effects before the initiation of the disease (82). Elevated estrogen levels during fetal life have been shown to influence morphology of the mammary gland (83), and are also thought to be responsible for the persistence of epithelial structures (terminal end buds) that are known to be sites of malignant transformation (84). Results from animal models and indirect human evidence indicate that exposure to elevated estrogen levels *in utero* may increase the risk for developing breast cancer in adulthood (83,85).

## 6.0 Coronary Heart Disease in Postmenopausal Women

Coronary heart disease is the leading cause of morbidity and mortality in women older than the age of 50 in the United States today. Traditional cardiovascular risk factors such as hyperlipidemia, glucose intolerance, and hypertension are more clearly associated with significant cardiovascular risk after menopause. The increased incidence of cardiovascular events in postmenopausal women and the evidence that cardiovascular disease on average manifests a decade later in women compared with men suggests that estrogen deficiency may predispose women to a higher cardiovascular risk.

Lipid profile changes after menopause include gradual increases in low-density lipoprotein cholesterol (LDL-C) and very low-density lipoprotein cholesterol (VLDL-C), which are thought to be secondary to the downregulation of the LDL receptors in the liver (86-88). Additionally, high-density lipoprotein cholesterol (HDL-C) levels decrease, and this change is a more powerful predictor of CHD mortality in postmenopausal women (89-90). Large prospective studies, including the Framingham Heart Study, documented a higher risk ratio of CHD in postmenopausal women with lower HDL-C levels (90-91).

There is evidence that lipoprotein(a) [Lp(a)] is also a significant risk factor for CHD in postmenopausal women and that HRT may have a benefit in lowering both LDL-C and Lp(a) (92). The Lp(a) particle is structurally similar to the LDL particle and plasminogen, and is also found in atherosclerotic lesions along injured arterial vessel walls. There is also evidence that Lp(a) may possess both prothrombic and proinflammatory characteristics (93,94). The Framingham Heart Study demonstrated that increased Lp(a) levels represented an independent cardiovascular risk factor for MI (95).

## 6.1 Hormone Replacement Therapy in Postmenopausal Women

Observations that the incidence of CHD events in postmenopausal women increases dramatically compared with premenopausal years led to the hypothesis that hormones are cardioprotective for women. It has been well documented that women with premature menopause, induced by bilateral oophorectomy, and without simultaneous HRT have a 2.2 times higher cardiovascular risk than premenopausal women of the same

age (96). The Nurses' Health Study (96) demonstrated an 8-fold increase in risk of CHD in women between the ages of 30 to 55 years with bilateral oophorectomy who did not receive concurrent estrogen replacement therapy.

Several biologic mechanisms for HRT-induced cardioprotection have been proposed including: improved lipid profiles, higher insulin sensitivity, and normal vascular reactivity. Early observational trials showed a significant decrease in cardiovascular events for women on HRT. However, recently published randomized clinical trial results have led to uncertainty about the cardiovascular benefits of HRT.

In May 2002, the data and safety monitoring board of the Women's Health Initiative (WHI) recommended early termination of the estrogen and progestin versus placebo arm of the study due to an unanticipated increase in invasive breast cancer (97). The board calculated a global risk index reflective of the relative risk of breast cancer versus coronary heart disease (CHD), nonfatal myocardial infarction (MI), and CHD death, along with secondary outcomes in the estrogen and progestin treated groups. This risk index showed a disproportionately increased risk over benefit in the HRT group. As a result, the investigators from WHI recommended that HRT should not be initiated or continued for primary prevention in postmenopausal women. Four years earlier, the Hormone and Estrogen Replacement Study (HERS) investigators showed no benefit of HRT in postmenopausal women with established CHD and possibly an increased risk of cardiovascular events within the first year of HRT administration (98). Primarily on the basis of this study, the American Heart Association (AHA) in 2001 delivered a consensus statement advising against starting HRT in women with a history of cardiovascular disease (99).

## 6.2 Effects of HRT during Menopause

As described above, HRT was thought to have a protective effect against CHD for almost 2 decades. Additionally, noncoronary benefits of HRT include alleviation of postmenopausal vasomotor symptoms, an increase in bone mineral density, and improvement of cognitive functioning (101). Oral HRT use is reported to increase HDL-C and to decrease LDL-C and Lp(a) by 10 to 15% (107). In the Postmenopausal Estrogen/Progestin Interventions (PEPI) Trial, also a randomized, double-blind, placebo-controlled study, women receiving 0.625 mg CEE had a 10 to 12% decrease in LDL over 3 years of treatment, and the addition of medroxyprogesterone acetate (MPA) or micronized progesterone (MP) did not attenuate this effect (108). The PEPI trial assessed the affect of various hormone regimens in postmenopausal women between the ages of 45 to 65 years. Patients treated with HRT had a 17 to 23% average drop in Lp(a) relative to placebo ($P < 0.001$), which remained significant at 3 years of follow-up. This effect was sustained in patients taking estrogen with and without concomitant progestin (108).

Recent data suggest that certain estrogen receptor polymorphisms may modify the effect of HRT on levels of HDL-C in postmenopausal women (109). In a substudy of the Estrogen and Replacement Atherosclerosis (ERA) trial, postmenopausal women with CHD and the estrogen receptor-α VS1-401c/c genotype (or a closely related genotype) had an increase in HDL subfraction 3 (HDL3) more than twice the amount observed in women of other genotypes. These differences were observed even after adjustment for age, race, diabetes, body mass index (BMI), and tobacco and alcohol consumption. Additionally, these changes were evident in women receiving both estrogen alone and estrogen with progesterone.

In addition to the lipid lowering effects of HRT, estrogen has been reported to act as an antioxidant (110), to decrease platelet aggregation and inhibit smooth muscle proliferation (111), and to improve overall endothelial function (112). In animal models, estrogen protects the endothelium and prevents myocardial dysfunction after brief episodes of ischemia and reperfusion (113). This beneficial effect of HRT on the endothelium is thought to be secondary to increased synthesis of NO (114), and the antiatherogenic effect of estrogen on the endothelium has been determined to be independent of serum lipid levels in animal models (115). Experimental animal studies have demonstrated that estrogen treatment reduced atherosclerosis by two thirds in injured rat aortas, and improved endothelial recovery in carotid arteries after balloon injury (116).

Other possible mechanisms of HRT benefit in this regard include a decrease in fibrinogen and plasminogen activating inhibitor, which increases fibrinolysis (117-118). Plasminogen activating inhibitor was decreased by 50% when conjugated equine estrogen (CEE) was given either alone or with progestin (118). The FINRISK Hemostasis study demonstrated significant decreases in fibrinogen concentration in postmenopausal women taking HRT compared with placebo (119-120). In the Atherosclerosis Risk in Community Study, 5436 postmenopausal women without CHD were given estrogen therapy (121). Results for the women taking estrogen versus nonestrogen users demonstrated an improved lipid and hemostatic profile, but no changes in the carotid intima–media thickness (117), suggesting that any reduction in cardiovascular disease in HRT users may not entirely be due to a slowing of the progression of atherosclerotic disease. Conversely, the Estrogen in the Prevention of Atherosclerosis Trial (EPAT), a

randomized, double-blind, placebo-controlled trial, showed that unopposed micronized 17β-estradiol reduced the subclinical progression of atherosclerosis regardless of the effect on lipid levels in healthy postmenopausal women (122). In fact, the greatest treatment benefit was observed in those women not on lipid-lowering therapy, suggesting no synergy with lipid-lowering therapies.

Although evidence exists for an increase in blood pressure with oral contraceptive therapy, HRT has a neutral effect on blood pressure in postmenopausal women (133). Because HRT has lower doses of estrogen compared with oral contraceptive pills, blood pressure is minimally affected. Reports have also described the effect of HRT on body weight and body fat distribution as neutral or favorable (124-125). HRT may also have a favorable benefit on insulin sensitivity (126-127). This is particularly important in the population of women who have dyslipidemia with hyperinsulinemia and visceral obesity, because many of these metabolic traits are typically interdependent. The Menopause Study showed a fall in fasting blood glucose and insulin levels, as well as an improvement in insulin reactivity after 1 year of HRT in postmenopausal women (126). However, not all studies have shown such a beneficial affect of estrogen on insulin sensitivity. Additionally, adding progestins to estrogen therapy may further decrease insulin sensitivity (128).

### 6.3 Noncardiac Risks and Benefits to HRT

The clinician and the patient are faced with a dilemma of balancing the beneficial effects of HRT with its possible adverse effects including breast cancer, uterine cancer, thromboembolic disease, and gallbladder disease. HRT has been shown to increase

breast cancer risk for each year of use, the risk being 1.35 (95% CI, 1.21–1.49) for women taking HRT for ≥ 5 years (129). The primary reason for prematurely stopping WHI in March 2002 was because of the increased incidence of invasive breast cancer (primary adverse outcome), which exceeded safety margins in women treated with estrogen and progestin (97). The overall hazard ratio for invasive breast cancer was 1.26 (CI, 1.00–1.59%), with the incidence increasing after 5 years. The hazard ratio was 2.13 (CI, 1.15–3.94%) for women taking prior HRT up to 5 to 10 years. The incidence of breast cancer in a randomized secondary prevention trial (HERS II) did not demonstrate any significant difference between treatment groups (130). The overall risk hazard with 95% CI for breast cancer in HERS and HERS II was 1.27 (95% CI, 0.84–0.94). However, breast cancer incidence was a secondary adverse outcome and the actual number of breast cancer deaths was only 3%, whereas lung cancer and colon cancer were 61% and 17% respectively, of the cancer deaths.

There is new evidence that HRT may decrease the effectiveness of breast cancer screening by mammography as a result of an increase in breast density (131). However, no clear relation exists with the type and duration of HRT and breast density changes on mammography. Most of the breast cancers reported with HRT use are typically less invasive, not involving deeper breast tissue or the lymph nodes (131). Even though a significant effect on breast cancer and subsequent mortality for postmenopausal women using HRT is rarely reported, physicians must still advise the patient about the unwanted effects of HRT. Other noncardiovascular risks of HRT evaluated in larger randomized trials included biliary tract surgery, ovarian cancer, endometrial cancer, and colorectal

Noncardiovascular benefits often considered when prescribing HRT include decreased incidence of fracture, and improved vasomotor and cognitive functioning. There are few randomized data available on HRT and prevention of cognitive decline or dementia. Treatment with HRT has not, however, been shown to delay Alzheimer's disease, even in the oldest group of women studied (135-136). Reduction of vasomotor symptoms is an accepted indication for use of HRT in postmenopausal women. When compared with other nonhormonal therapies such as venlafaxine, paroxetine, or clonidine, estrogen is more effective in the treatment of hot flashes (136-137). Many women have severe symptoms, especially after bilateral oophorectomy, and require a higher dose estrogen regimen. Another noncardiac benefit is prevention of osteoporosis (108,138). Women randomized to HRT have 5 to 7% more bone mass after 2 to 3 years of treatment than women taking placebo. Until recently, no randomized trial actually showed a reduction in osteoporotic fractures. The WHI is the first definitive trial showing a reduction in fractures of the hip, vertebrae, and other areas (97).

## 7.0 Selective Estrogen Receptor Modulators (SERMs)

In 1936, Antoine Lacassagne presented a paper at the American Association for Cancer Research that recommended estrogen as a potential target for the prevention of breast cancer in healthy women (139). At that time, however, the target for hormone action was unknown and there were no antagonists for estrogen action. It was not until 1962 that Jensen et al. (140) first described the estrogen receptor (ER) in estrogen-responsive tissues, and applied this knowledge to establish a predictive test to identify breast tumors that would respond to endocrine therapy (141). In 1958, Lerner et al.

reported the pharmacological properties of the first nonsteroidal estrogen antagonist, MER 25. MER 25, a complete antiestrogen with no other hormonal or antihormonal activities, was not developed for clinical use because of low potency and unacceptable side effects (142).

The study of the nuclear receptor superfamily has provided hope that enhanced basic understanding can lead to new treatments in the clinic. It has long been known that nuclear receptors play important roles in disease, including cancer, heart disease, diabetes, obesity, and osteoporosis. A great deal of attention has been directed to the role of steroid receptors in breast (estrogen receptor) and prostate (androgen receptor) cancer, as well as osteoporosis. This emphasis has led to research efforts aimed at better understanding the responses of specific tissues to the steroid receptors, as well as to develop ligands designed to modulate those responses.

As previously discussed estrogen and estrogen/progestin have been used to delay or reverse some of the changes related to menopause, however, epidemiologic and clinical research have elucidated potential long-term risks associated with such therapy. The has led to increased research aimed at developing compounds that elicit important and tissue-specific functions of the steroid receptors without their deleterious effects. This class of compounds has been named selective receptor modulators (SRM). The term Selective Estrogen Receptor Modulators (SERMs) has been used to describe a group of pharmaceuticals that manifest estrogen receptor (ER) agonist activity in some tissues, but that oppose estrogen action in others. The SERMs are chemically diverse compounds that

lack the steroid structure of estrogens (see Figure 4) but possess a tertiary structure that allows them to bind to the estrogen receptor.

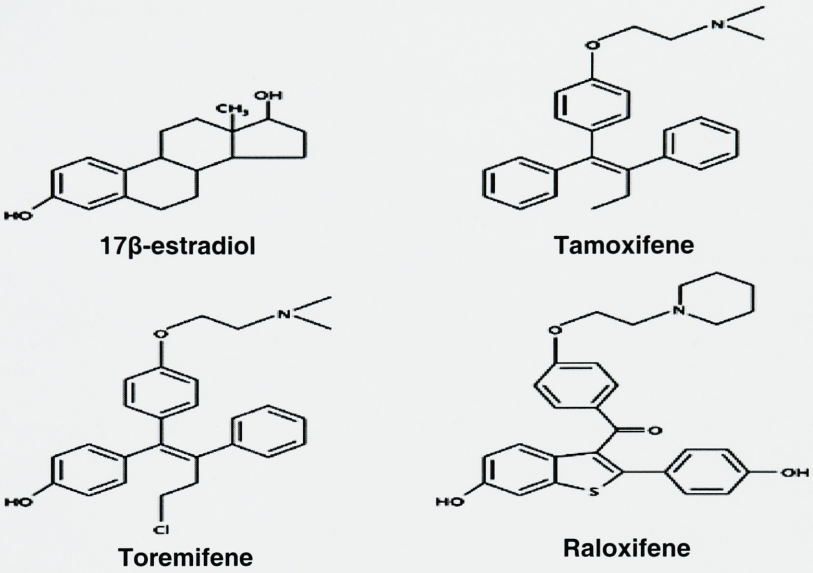



**Figure 4 - Chemical Structure of 17β-Estradiol, the Main Physiologically Relevant Estrogen, and Three SERMs Approved by the Food and Drug Administration.** 17β-Estradiol has a cyclophenanthrene structure, whereas tamoxifen and toremifene have a triphenylethylene structure and raloxifene has a benzothiophene structure. Although the primary structure of these SERMs differs strikingly from that of estrogens, they have a conformation that allows them to bind to the ligand-binding domain of the estrogen receptor. (From Riggset al., Selective Estrogen-Receptor Modulators -- Mechanisms of Action and Application to Clinical Practice., N Engl J Med 2003 348: 618-629)

Tamoxifen was the first SERM to be used in clinically. Because of the clinical success of tamoxifen use in breast cancer, other SERMs have been developed for use in breast cancer. When positive effects of some SERMs were also noted in bone, further development of second and later generations of these drugs accelerated. The operative

concept has been to develop compounds that drive the targeted receptor to a form that allows for tissue- and gene-specific action. The task now before us is to better define where SERMs may be useful, how best to use them, and how to develop even more specific compounds with important therapeutic applications.

Tamoxifen was the first drug to be classified as a SERM. Tamoxifen blocks the binding of estradiol to the human ER and is effective in controlling the growth of ER-positive, but not ER-negative, breast tumors (143-144). Tamoxifen was, however, found to act as an estrogen agonist in bone and the uterus of an ovariectomized rat model (145-147), despite having well-defined antiestrogenic/antitumor actions in the mammary gland (148-151). Indeed, the transplantation of breast and endometrial tumors into athymic mice treated with tamoxifen results in decreased breast cancer growth, but an increase in endometrial cancer growth (152). To explain these findings, it was proposed that the tamoxifen-ER complex acts as an agonist at some sites, but as an antagonist at other select sites (150-152). Tamoxifen treatment maintains bone density in postmenopausal women (153) and lowers circulating cholesterol concentrations (154-155). Like estrogen, tamoxifen lowers LDL cholesterol levels and protects LDL from oxidation; but unlike estrogen, it does not increase levels of the antiatherogenic HDL (156). The differential action of tamoxifen on breast versus endometrial cancer growth has also been observed in clinical trials showing reduction in the incidence of contralateral breast cancer, but an increase in the incidence of endometrial cancer (157).

## 7.1 Mechanisms of Action of SERMs

In the classic model of hormonal action, the unoccupied estrogen receptor resides in the nucleus of the target cell in an inactive form. Binding to an agonist, such as estradiol, alters the structure and properties of the estrogen receptor, allowing the receptor dimer to interact with specific DNA sequences (estrogen response elements) within the promoters of responsive genes (158). The DNA-ER complex may then regulate gene transcription, either positively or negatively as shown in Figure 5. However, the

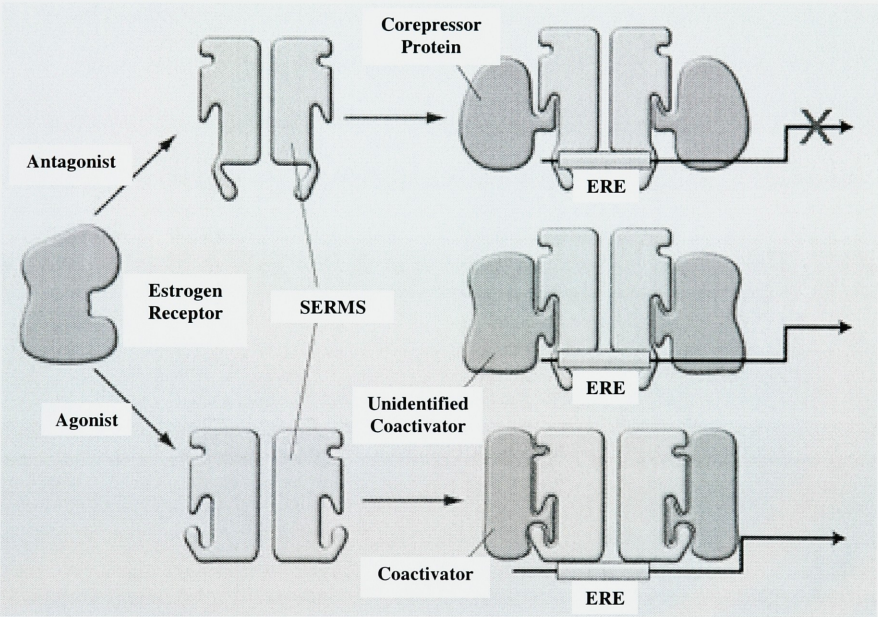



**Figure 5 – Estrogen Receptor Action.** On binding an agonist or an antagonist, the estrogen receptor (ERα or ERβ) undergoes a conformational change that permits its dimerization and facilitates the interaction of the dimer with estrogen response elements (EREs) located within target genes. It has been determined that estrogen facilitates the interaction of the estrogen receptor with coactivators. An antagonist-activated estrogen receptor, on the other hand, interacts preferentially with a corepressor protein. The binding of different SERMs to the receptor permits it to adopt conformational states distinct from that induced by classic agonists or antagonists. The weight of available evidence suggests that the structure of some SERM–estrogen-receptor complexes favors corepressor recruitment and that of others favors some affinity for known coactivators. Some SERMs may also facilitate the interaction of the estrogen receptor with yet-to-be-identified coactivators with which it would not normally couple. The implication of this model is that SERM activity will be influenced by the relative levels of expression of the cofactors (corepressors and coactivators) in target cells. (From Riggset et al., Selective Estrogen-Receptor

Modulators -- Mechanisms of Action and Application to Clinical Practice., N Engl J Med 2003 348: 618-629)

recognition that tamoxifen and other SERMs have tissue-specific agonist–antagonist activity suggested that the classic model was incomplete and that estrogen action was more complex than had been thought (159-160).

### 7.1.1 Differential Estrogen-Receptor Expression in a Given Target Tissue and Differential Estrogen Receptor Localization in Specific Cells

Target cells for estrogen action contain varying concentrations of homodimers of one or both of two species of estrogen receptors — ER-α and ER-β — as well as ER-α/ER-β heterodimers. In addition to variable expression of the two ER isoforms in target tissues, there is also variable localization of the ERs within a specific cell. Although some of the effects of estrogen are mediated by intracellular ERs that act as ligand-activated transcription factors regulating gene expression, the non-genomic effects of estrogen (such as the rapid increase in nitric oxide release in endothelial cells) are mediated by an ER signaling system that is localized to cell membrane caveolae (26). Table 1 shows the target tissues and the rapid non-genomic cellular signaling pathways activated by the two ER isoforms.

ER-α is almost always an activator, whereas ER-β can inhibit the action of ER-α by forming a heterodimer with it (161-162). A microarray analysis in mice with deletions of ER-α or ER-β showed that ER-β inhibited the transcription of 240 estrogen-responsive genes by 46 percent (163). Thus, the degree of expression of these two receptor isoforms

| Receptor | Rapid Action | Cell Types used |
|---|---|---|
| **ERα** | Ca2+ mobilization | Mammary Carcinomas |
| | Activation of ERK1 and ERK2 | a. Neuroblastoma<br>b. Mammary carcinoma<br>c. Osteoblasts<br>d. Primary Cortical neurons<br>e. Cortical explants<br>f. Bone cell line |
| | Activation of G proteins | Ovary |
| | Inositol phosphate production | Ovary |
| | Stimulation of Adenylyl Cyclase | Ovary, breast, uterus |
| | Inhibition of JNK | Ovary |
| | Activation of IGF1 | Kidney |
| | Activation of PI3K | Endothelium, Breast |
| | Activation eNOS | Endothelium |
| | Activation Akt | Breast, Endothelium |
| | | |
| **ERβ** | Activation of ERK1 and ERK2 | Ovary and Breast |
| | Activation of JNK | Ovary |
| | Inositol phosphate production | Ovary |
| | Activation of G proteins | Ovary |
| | Stimulation of Adenylyl Cyclase | Ovary |

**Table 1 – Cellular signaling pathways activated by the two ER isoforms in various target tissues** (Adapted from Cato et al., Rapid Actions of Steroid Receptors in Cellular Signaling Pathways, Sci. STKE 2002 (138) re9).

will affect the cellular responsiveness to estrogens (164-165). Since SERMs bind to both ER isoforms, these drugs affect the cellular responsiveness as well. Indeed, SERMs such as raloxifene and tamoxifene function as pure antagonists when acting through ER-β but can function as partial agonists when acting through ER-α (165).

### 7.1.2 Differential Estrogen-Receptor Conformation on Ligand Binding

Protein crystallography and techniques that evaluate protein surface conformational changes have shown that binding of the ER by estradiol, tamoxifen, raloxifene, or the pure estrogen antagonist ICI 164,384 results in a unique estrogen-receptor conformation for each ligand (166-168). Thus, the ligand binding results in various estrogen-receptor conformations that range from that assumed when the receptor is bound to estrogens at one extreme to that assumed when the receptor is bound to antiestrogens at the other extreme. Binding of SERMs to ER results in a continuum of intermediate shapes (168-169).

### 7.1.3 Differential Expression and Binding to the Estrogen Receptor of Coregulator Proteins

More than 20 coregulator proteins have been discovered that bind to estrogen receptors and modulate their function, each acting as either a positive or a negative transcriptional regulator (a coactivator or a corepressor, respectively) (170-173). Depending on the unique receptor conformation induced by ligand binding, varying combinations of coregulator proteins interact with the estrogen receptor and modulate its function in a variety of ways. The relative and absolute levels of expression of

coregulator proteins vary among estrogen target cells (171). In a study by Shang and Brown (174), it was found that tamoxifen and raloxifene, which are estrogen antagonists in the breast, act on mammary cells by recruiting corepressors to estrogen-receptor target promoters. In contrast, tamoxifen, which is an estrogen agonist in the endometrium, acts by recruiting coactivators to the estrogen-receptor target promoters. This recruitment does not occur with raloxifene, which has no effect on the endometrium. It was also found that the tamoxifen/ER-α complex activates transcription by indirectly binding to promoters that do not contain estrogen response elements through protein-to-protein contacts with other DNA-bound transcription factors. Moreover, the agonist effect of tamoxifen is dependent on a higher concentration of a key coactivator, steroid receptor coactivator-1 (SRC-1), in the endometrial cells. Thus, variable local concentrations of different coregulator proteins may contribute to the tissue-selective pharmacology of SERMs.

### 7.2 SERMS and the Cardiovascular System

One consequence of the Women's Health Initiative findings has been an increased interest in therapy with SERMs, because of their potential to retain most of the beneficial effects of estrogen while avoiding most of its adverse effects. While the SERMs are similar to conventional estrogen replacement therapy in many ways, there are also differences with potentially important cardiovascular implications.

### 7.2.1 The Effects Raloxifene on Cardiovascular Risk Factors

Raloxifene inhibits the growth of estrogen receptor-dependent mammary tumors and reduces the occurrence of nitrosomethylurea-induced mammary tumors in rats. It has been classified as a SERM on the basis of studies in which it prevented bone loss and lowered serum cholesterol levels without stimulating proliferation of the endometrium (175).

### 7.2.2 Raloxifene Effects on Lipoproteins and Lipids

Delmas et al. (176) studied the effect of raloxifene on serum lipids (among other end points) in 601 postmenopausal women who were randomly assigned to receive 30, 60, or 150 mg of raloxifene or placebo daily for 24 months. Serum concentrations of total cholesterol and LDL cholesterol decreased in all raloxifene treatment groups, whereas serum concentrations of HDL cholesterol and triglycerides did not change.

In a study by Walsh et al. 390 postmenopausal women were enrolled in a randomized, placebo-controlled clinical trial (177). These women were randomly assigned to treatment with raloxifene (60 mg or 120 mg), HRT (equine estrogen 0.625 mg and medroxyprogesterone 2.5 mg), or placebo. Lipids and coagulation factors were measured after daily treatment for 6 months. The study showed that raloxifene favorably alters several markers of cardiovascular risk in healthy postmenopausal women. While many of its effects are similar to those of conventional HRT, there are also important differences. Raloxifene lowered low-density lipoprotein cholesterol levels similarly to estrogen. However, raloxifene lacked the potentially beneficial effects of HRT on high-

density lipoprotein cholesterol levels and plasminogen activation inhibitor-1, as well as the potentially adverse effects of HRT on triglycerides and C-reactive protein. Raloxifene also had a potentially beneficial fibrinogen-lowering effect not seen with conventional HRT. The net effect of these differences is not clear. Proof that raloxifene reduces the risk of heart disease must await the results of ongoing clinical trials with cardiovascular event end points.

### 7.2.3 Raloxifene Effects on Atherosclerosis in Animal Models

In a study by Bjarnason et al. (179), ovariectomized rabbits treated with raloxifene and 17β-estradiol had two-thirds and one-third, respectively, of the extent of aortic atherosclerosis (as evaluated by the cholesterol content of the aorta) compared with the placebo group. However, Clarkson et al. (180) treated ovariectomized cynomolgus monkeys fed an atherogenic diet with raloxifene (1 or 5 mg/kg/day), or CEE at a dose estimated to mimic 0.625 mg/day CEE in women, or placebo. Treatment with CEE resulted in an approximately 70% reduction in coronary artery plaque size relative to that in the placebo group, whereas neither the low nor the high dose of raloxifene had an effect on coronary artery plaque size that differed from placebo-treated animals. Although raloxifene appears to lower cholesterol content of the aorta in the study by Bjarnason et al., this SERM may not have a considerable effect on LDL or apolipoprotein B levels which are also known to contribute to coronary artery plaque formation.

### 7.2.4 Effects of Estrogen and Raloxifene on Markers of Inflammation in Postmenopausal Women

When cell adhesion molecules are expressed on the surfaces of endothelial or mononuclear cells in culture following cytokine stimulation, they are shed into the supernatant within 24 hours. These cell adhesion molecules are also measurable in the sera of humans. The pathophysiologic relevance of soluble cell adhesion molecules has been suggested by their localization in atherosclerotic plaques (181), higher serum levels in patients with atherosclerosis relative to control subjects (182), and association with increased risk of myocardial infarction in apparently healthy (male) subjects (183). Serum concentrations of E-selectin, ICAM-1, and VCAM-1 were reported to be higher in postmenopausal women with coronary artery disease who were not taking HRT than those taking HRT at the time of cardiac catheterization (184). In a study of hypercholesterolemic postmenopausal women Koh et al. (185) reported that CEE significantly reduced circulating levels of the cell adhesion molecules E-selectin, ICAM-1, and VCAM-1 relative to respective pretreatment values, with the greatest effect noted on E-selectin, the cell adhesion molecule specific to the activated endothelium.

Blum et al. (186) conducted a randomized, double-blind, three-period crossover treatment trial to examine whether raloxifene versus CEE reduces serum levels of inflammatory markers in 23 postmenopausal women. Levels of C-reactive protein (CRP), a marker of increased cardiovascular risk, were increased by 50% with CEE, but were unchanged with raloxifene versus placebo values. Levels of interleukin-6 (IL-6), a cytokine implicated in atherosclerosis that also conveys increased risk, were

nonsignificantly increased with CEE and raloxifene relative to placebo. Levels of matrix metalloproteinase-9 (MMP-9) (an enzyme secreted by macrophages and activated smooth muscle cells and implicated in plaque rupture) were increased by 30% with CEE relative to placebo, but not with raloxifene. Serum E-selectin levels were reduced by both CEE and raloxifene compared with placebo, with CEE having a greater effect than raloxifene. Both therapies equally reduced serum ICAM-1 levels compared to placebo. Serum VCAM-1 was reduced with CEE, but raloxifene did not have an effect on VCAM-1 when compared with placebo. Thus, raloxifene lowers circulating levels of some cell adhesion molecules to a lesser extent than CEE in otherwise healthy postmenopausal women, but does not increase other markers of inflammation as seen with CEE therapy (CRP, interleukin-6, and MMP-9). The biologic relevance of these differences in effects on markers of inflammation remains to be determined in clinical trials.

The ultimate goal of SERM research is the discovery of a tissue-selective drug that has all the beneficial effects of estrogen and has none of its adverse effects as shown in Figure 6. The present study was undertaken to evaluate a panel of novel SERMs with regard to their effects on vascular endothelial and breast cancer cells. In collaboration with Eli Lilly pharmaceuticals, we received 10 potential SERMs for which we established dose response curves for proliferation of immortalized vascular endothelial cells (EA.hy926) and human breast cancer cells (MCF-7). We also evaluated the rapid induction of eNOS activation in the EA.hy926 endothelial cells. This cell line has been used in the Bender lab and has been reported (187) to express a 46-kDa protein that immunoreacts with selective ER-alpha Abs and is capable of tranducing $E_2$-triggered rapid signaling. EA.hy926 cells phenotypically represent fully differentiated vascular

ECs that exclusively express ER46 under our culture conditions. These cells are highly responsive to membrane-impermeant $E_2$ within minutes, as demonstrated by NO release and cGMP production through an $E_2$-stimulated, ligand-dependent PI3-kinase/Akt/endothelial NO synthase (eNOS) pathway (188,189). In addition, we examined the effects of this group of SERMs on MCF-7 breast cancer cell apoptosis.

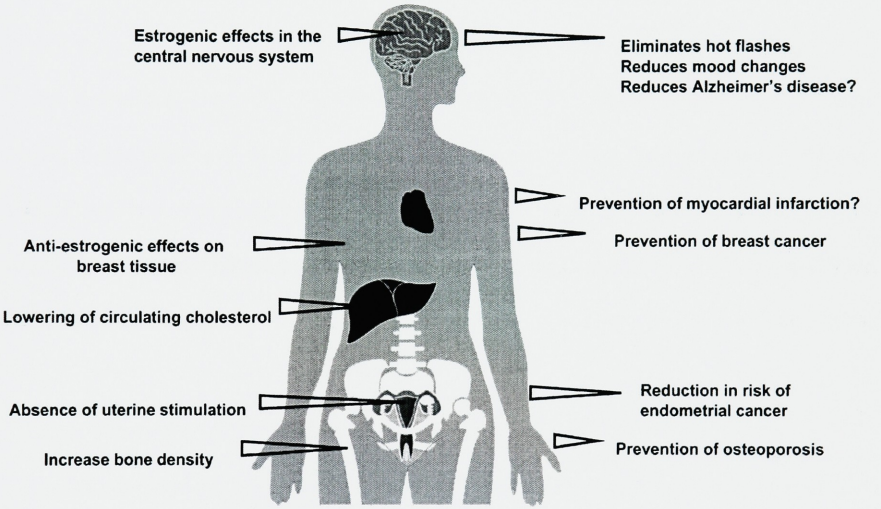



**Figure 6 – The Ideal SERM**. Estrogen is associated with decreases in osteoporosis, and there are unconfirmed beneficial effects with estrogen in preventing Alzheimer's disease and coronary heart disease. The principal positive action of estrogen is to alleviate menopausal symptoms and mood changes. The negative actions of estrogen are an increased risk of breast or endometrial cancer. An ideal SERM would enhance the benefits of estrogen but would prevent breast and endometrial cancers. In the latter case, progestin therapy would be unnecessary, and periodic menstrual bleeding would be avoided. Raloxifene possesses this property. To date, tamoxifen has been shown to decrease breast cancer risk, and raloxifene has been shown to reduce the risk of fractures. Neither tamoxifen nor raloxifene fulfills the criteria for an ideal SERM, but continuing clinical evaluation will establish the long-term safety of the SERM concept. (From Craig et al., Selective Estrogen Receptor Modulation and Reduction in Risk of Breast Cancer, Osteoporosis, and Coronary Heart Disease, J Natl Cancer Inst (2001); **93**: 1449-1457.

## 1.0 Material and Methods

### 1.1 Materials

Stock solutions of LY compounds (provided by Lilly Pharmaceuticals) were prepared in DMSO with final DMSO concentrations less than 0.1%. 17ß-estradiol was purchased from Sigma. The anti-eNOS antibody was purchased from Transduction Laboratories. Anti-phosphorylated Akt (pAKT) and anti-Akt antibodies were purchased from Cell Signaling. The anti-phosphorylated eNOS (peNOS) antibody was from New England Biolabs. Secondary antibodies, anti-rabbit horseradish peroxidase and anti-mouse horseradish peroxidase, were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA. The CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay was purchased from Promega. Rainbow molecular weight markers were purchased from Amersham Pharmacia.

### 1.2 Cell lines and culture conditions

Breast adenocarcinoma MCF-7 cells (Michigan Cancer Foundation, Detroit, MI) were propagated in phenol-free M199 medium supplemented with 10% gelding horse serum (GHS) (<1.0 pg/mL estradiol), insulin (10ug/ml), L-Glutamate 2mM, penicillin 100U/ml and streptomycin 100$\mu$g/ml. The cell line was passaged in phenol-free M199 medium, subcultured every 6 days with a split ratio of approximately 1:10, and growth medium was changed every 2 days. Before $E_2$ or LY stimulation, cells were cultured in "$E_2$-free medium" which consisted of phenol-free M199 with 0.5% GHS, L-Glutamate 2mM, penicillin 100U/ml, and streptomycin 100$\mu$g/ml. "Starvation medium" consisted

of phenol-free M199 with L-Glutamate 2mM, penicillin 100U/ml, streptomycin 100$\mu$g/ml and BSA 0.25%.

The permanently established EA.hy926 endothelial cell (EC) line (200) was provided by CJS Edgell (University of North Carolina). Cells were maintained in DMEM, which was supplemented with 10% FBS, 5 mmol/L hypoxanthine, 0.8 mmol/L thymidine, 20 $\mu$mol/L aminopterin, L-Glutamate 2mM, penicillin 100U/ml and streptomycin 100$\mu$g/ml. Before $E_2$ stimulation, cells were cultured in "$E_2$-free deprivation medium", which consisted of phenol-free DMEM with gelding horse serum (<1.0 pg/mL estradiol), L-Glutamate 2mM, penicillin 100U/ml, streptomycin 100$\mu$g/ml. "Starvation medium" consisted of phenol-free DMEM, L-Glutamate 2mM, penicillin 100U/ml, streptomycin 100$\mu$g/ml and BSA 0.25%.

**1.3 MCF-7 Cell Proliferation Assay**

MCF-7 cells cultured in C-100 Petri dishes were harvested using 0.25% trypsin when approximately 85% confluent. MCF-7 cells were resuspended in culture media and seeded in 96-well microtiter plates, at a concentration of 8000 cells/well in a volume of 0.2ml of culture media. The cells were incubated at 37$^0$C for 24hrs before the culture media was aspirated from the wells. The cells were washed twice with CMF-PBS, and 0.2ml of "$E_2$-free deprivation medium" were added followed by incubation at 37$^0$C for 48hrs. The cells were then washed again twice with CMF-PBS and 0.2ml of "starvation medium" was added for 12hrs. After the starvation period the various cell treatments were prepared in "$E_2$-free deprivation medium" and added to the appropriate wells. The cells were incubated at 37$^0$C for 48hrs before measurements were made.

Working concentrations of compounds LY1-11 were prepared the day of the experiment in 100% ETOH as dilutions from concentrated stocks in DMSO maintained at -80$^0$C. Solvent controls had the same concentration of ETOH and were added to all the experiments.

Dose response curves for the 11 potential SERM compounds were established using the CellTiter 96® $AQ_{ueous}$ Non-Radioactive Cell Proliferation Assay (Promega). This is a calorimetric method for determining the number of viable cells in proliferation assays. The CellTiter 96® $AQ_{ueous}$ Assay is composed of solutions of a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine methosulfate) PMS. MTS is bioreduced by cells into a formazan product that is soluble in tissue culture medium. The absorbance of the formazan product is measured directly from 96-well assay plates without additional processing. The conversion of MTS into the aqueous soluble formazan product is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product, as measured by absorbance at 490nm, is directly proportional to the number of living cells in culture. The 96-well microtiter plates were read on a microtiter plate reader (Molecular Devices Ltd., Crawley, West Essex, United Kingdom).

**1.4 Endothelial Cell Proliferation Assay**

EA.hy926 ECs cultured in C-100 Petri dishes were harvested when confluent. ECs were resuspended in "$E_2$-free deprivation medium" and seeded in 96-well microtiter plates, at a concentration of 8000 cells/well in a volume of 0.2ml of culture media. The

cells were incubated at 37$^0$C for 48hrs before the culture medium was aspirated from the wells. The cells were washed twice with CMF-PBS and 0.2ml of "$E_2$-free deprivation medium" added followed by incubation at 37$^0$C for 48hrs. The cells were then washed again twice with CMF-PBS and 0.2ml of "starvation medium" added for a 12hr starvation period. After the starvation period the various LY treatments for the cells were prepared in "$E_2$-free deprivation medium" and added to the appropriate wells. The cells were incubated at 37$^0$C for 48hrs before measurements were made using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega).

**1.5 MCF-7 Cell Cycle Progression Assay**

MCF-7 cells propagated in "$E_2$-free deprivation medium" in C-100 Petri dishes were used when approximately 75% confluent. The plates were washed with CMF-PBS and 8ml/plate of starvation media added, followed by incubation at 37$^0$C for 12hrs. After the starvation period the various cell treatments were prepared in starvation media and added to the appropriate plates. The cells were incubated at 37$^0$C for 48hrs.

**Preparation of Cells for Fixation and Cell Cycle Analysis**

The plates were washed three times with CMF-PBS and trypsinized. The cells were then resuspended in 0.750ml ACCUMAX™ (cell detachment reagent), transferred to 1.5ml eppendorf tubes and incubated at 37$^0$C for 15 min. The tubes were then spun at 8000rpm for 3 min and the supernatant aspirated. The cell pellet was resuspended with 0.5ml cold 70% ETOH and incubated at -20$^0$C for one hour. The cells were spun at 8000rpm for 3 min and the supernatant aspirated. The cell pellet was resuspended in

ACCUMAX™ and incubated at $37^0$C for 10 min. The cells were then spun again at 8000rpm for 3 min and the supernatant aspirated. The cell pellet was resuspended in 0.2ml of staining buffer (20$\mu$g/ml propidium iodide, 0.05% Triton X-100, 200$\mu$g/ml RNase in CMF-PBS) and placed on ice for 45 min. 1ml of washing buffer (1%BSA, 0.03% NaN3 in CMF-PBS) was then added and the cells were spun at 8000rpm for 3 min and the supernatant aspirated. Finally, the cells were resuspended in 0.5ml of FACS assay buffer (1% formaldehyde, 1% BSA in CMF-PBS). DNA content of 10,000 events(cells)/treatment was analyzed using a FACSCalibur™ flow cytometer (Becton Dickinson) and the CellQuest software version provided by the manufacturer.

**1.6 Western Blot Analysis**

Cells were lysed as follows. Cell monolayers were washed twice in ice-cold phosphate-buffered saline then scraped into ice-cold lysis buffer (in mmol/L) Tris-HCl 20 (pH 7.4), EDTA 2.4, Triton X-100 1%, sodium deoxycholate 1%, SDS 0.1%, NaCl 100, NaF 10, $Na_3V0_4$ 1, $NaPiPO_4$ 1, and protease inhibitor cocktail (Roche).). The lysates were incubated for 5 min on ice, and cellular debris was cleared by centrifugation (15,000g, 5 min, 4 °C). Equal amounts of total protein were separated by SDS-PAGE then transferred to nitrocellulose. Proteins were visualized using enhanced chemiluminescence after incubation (overnight at 4 °C for primary and 1 hr at room temperature for secondary antibodies) using the following primary antibodies: Anti-eNOS, Anti-phosphorylated eNOS, Anti-phosphorylated Akt and anti-Akt. Immunoblots were probed with species-specific secondary antibodies coupled to horseradish peroxidase before visualized by enhanced chemiluminescence.

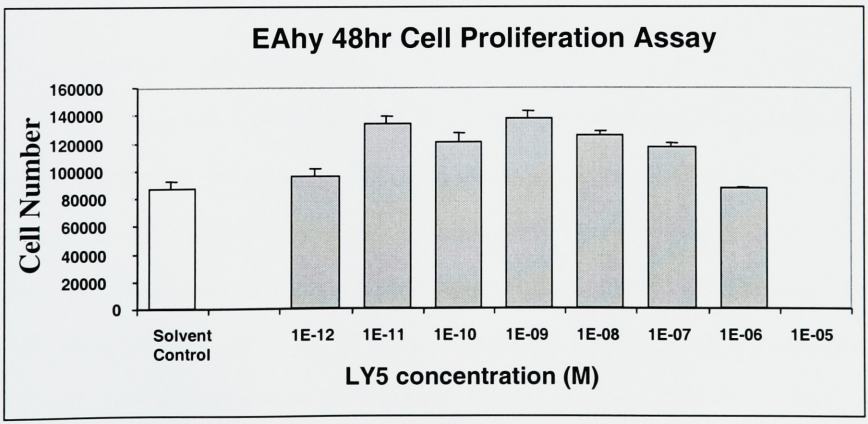
## Results

The results below (1-4) are those of the LY compounds that were chosen based on the results of the cell proliferation screening assays performed on EA.hy926 endothelial cells and the MCF-7 breast cancer cells. The results of the initial screening assays revealed general patterns amongst the compounds in terms of their effects on endothelial and epithelial cell proliferation that are represented by the four chosen compounds. These four compounds were used to further examine the effects on MCF-7 cell cycle analysis, and to evaluate endothelial cell p-Akt and p-eNOS expression using western blot analysis.

### 1.0 Results for compound LY5

### 1.1 EA.hy926 Cell Proliferation Assay

Stimulation of endothelial cells with LY5 showed a bell-shaped curve of efficacy, with a decreasing stimulatory effect at the highest concentration tested. Treatment with LY5 $10^{-12}$M resulted in cell proliferation comparable to control. Higher concentrations of LY5 ($10^{-11}$M to $10^{-7}$M) resulted in an increase in endothelial cell proliferation when



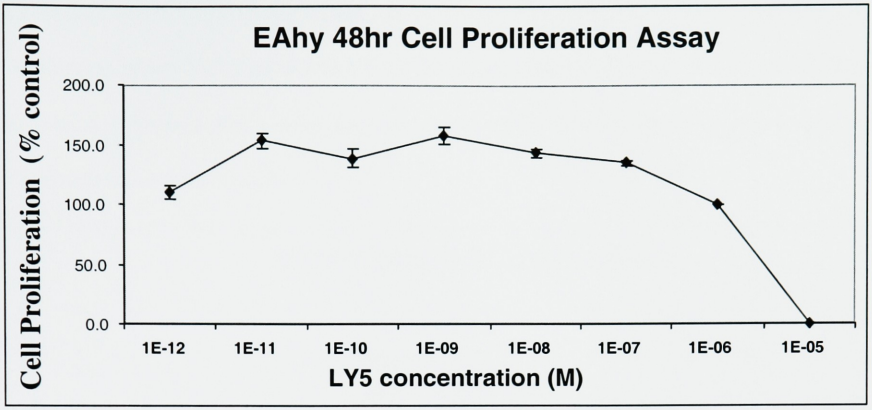



**Figure 1 - Cell proliferation Assay with EA.hy926 endothelial cells.** Multi-well dishes plated in triplicate, 8000 cells/well. The cells were estrogen deprived for 48 hours, followed by a 12 hour starvation period before stimulation with LY 5 at the concentrations indicated above. The plates were read 48 hours after stimulation.

compared to control as shown in Figure 1. Further increase in LY5 concentration to $10^{-6}$M resulted in a decrease in cell proliferation down to a level similar with control, and at LY5 $10^{-5}$M there is a precipitous drop in cell proliferation, with no viable cells present after 48hrs, suggesting that LY5 may have a toxic or an anti-estrogenic effect at higher concentrations.

### 1.2 MCF-7 Cell Proliferation Assay

Treatment with LY5 resulted in a bell-shaped curve of efficacy, with a peak level of cell proliferation observed at $10^{-11}$M. Stimulation with LY5 $10^{-12}$M resulted in a 40% decrease in cell proliferation compared to control, however, stimulation with LY5 $10^{-11}$ produced a 2-fold increase in cell proliferation when compared to the lower concentration. Stimulation with LY5 $10^{-11}$M and $10^{-10}$M also resulted in cell proliferation approximately 1.2 fold above the level seen with control. Further increases in LY5 concentration inhibited cell proliferation compared with control, with a 40% decrease for LY5 at $10^{-6}$M,

suggesting an anti-estrogenic effect on MCF-7 cells at these higher concentrations. Stimulation of MCF-7 cells with LY5 $10^{-5}$M resulted in a 70% drop in cell proliferation compared to control which may suggest a toxic effect in addition to an anti-estrogenic effect at this concentration.

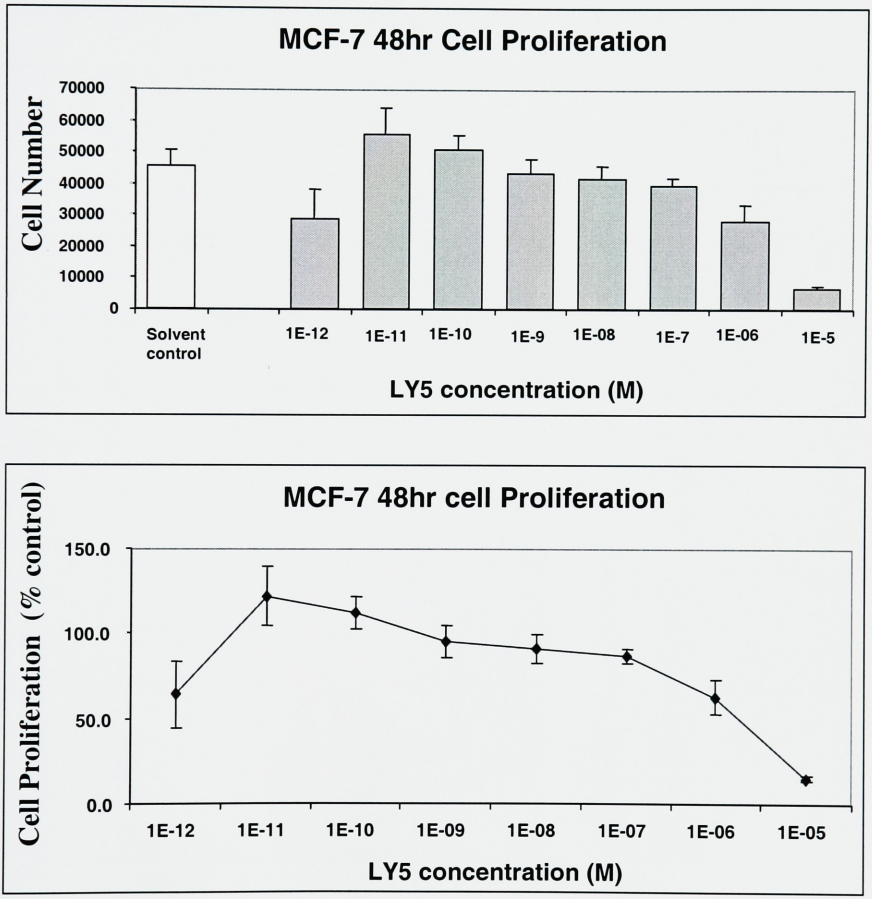



**Figure 2 - Cell proliferation Assay with MCF-7 breast cancer cells.** Multi-well dishes plated in triplicate, 8000 cells/well. There was a 12 hour starvation period before stimulation with LY5 at the concentrations indicated above. The plates were read 48 hours after stimulation.

### 1.3 MCF-7 Cell Cycle Analysis For Estrogen and Insulin

MCF-7 cells cultured in prolonged estrogen deprived media, were serum starved for 12 hours followed by a stimulation period. The optimal conditions for MCF-7 cell cycle analysis were established and it was found that the greatest percentage of cells in S and G2/M phases were achieved after 48 hours of stimulation with insulin or estrogen as shown in Figure 3 below. The optimal estrogen concentration for stimulation was 1 ng/ml as shown by the 2-fold increase in S phase population and the 2.5-fold increase in G2/M phase population of MCF-7 cells, as shown in Figure3. As expected, estrogen and insulin confer a significant protective advantage against apoptosis compared with control

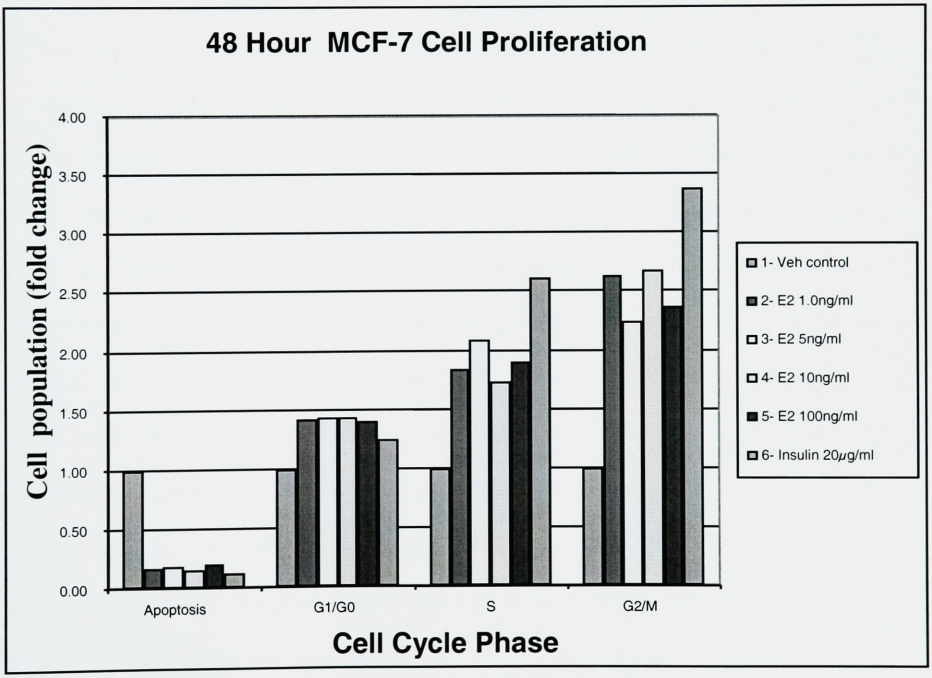



**Figure 3 – MCF-7 Cell Cycle Analysis.** MCF-7 cells cultured in prolonged estrogen deprivation were serum starved for 12 hours and stimulated with insulin (20μg/ml) and varying concentrations of estrogen.

(190), as shown by the 70% decrease in the percentage of apoptotic cells during cell cycle analysis. The protective effect against apoptosis does not appear to depend on the concentration of estrogen used to stimulate the breast cancer cells in the range of concentrations examined in the above experiment (1 to 100ng/ml).

### 1.3.1 MCF-7 Cell Cycle Analysis for LY5

Cell cycle analysis was performed on MCF-7 cells stimulated with estrogen and/or LY5. LY5 $10^{-12}$M resulted in a four-fold increase in the population of apoptotic cells, a 40% decrease in the G1/G0 phase and a no significant change in the population of S and G2/M phase cells when compared to estrogen. This suggests that LY5 decreases the survival of breast cancer cells as evident by the decrease in the G1/G0 population and the increase in apoptotic cells when compared to estrogen. LY5 $10^{-6}$M resulted in a similar increase in the population of apoptotic cells compared to LY5 $10^{-12}$M, however, there was also a 20% decrease in the population of S and G2/M phase cells when compared to estrogen and LY5 $10^{-12}$M. This suggests that LY5 $10^{-6}$M acts to decrease the survival of MCF-7 cells (perhaps due to an increase in apoptosis), but also acts to decrease the percentage of cells progressing to the S and G2 phases of the cell cycle, as compared to estrogen. LY 5 $10^{-9}$M caused a significant decrease in the percentage of apoptotic cells, when compared to the higher and lower concentrations of LY5 ($10^{-12}$M and $10^{-6}$M). Thus, the results of MCF-7 cell cycle analysis are consistent with the results for the MCF-7 cell proliferation assays, which produced a bell-shaped curve in response to increasing concentrations of LY5. LY5 $10^{-12}$M resulted in an increase in apoptotic cells during cell cycle analysis, reflected by a decreased cell numbers observed in the cell proliferation assay. LY5 $10^{-9}$M resulted in a decrease in apoptotic cells during cell cycle

analysis, reflected by an increase in cell numbers observed in the cell proliferation analysis. LY5 $10^{-6}$M resulted in an increase in the population of apoptotic cells in addition to a decrease in the percentage of cells in the S and G2/M phases, reflected by a decrease in cell numbers in the cell proliferation assay.

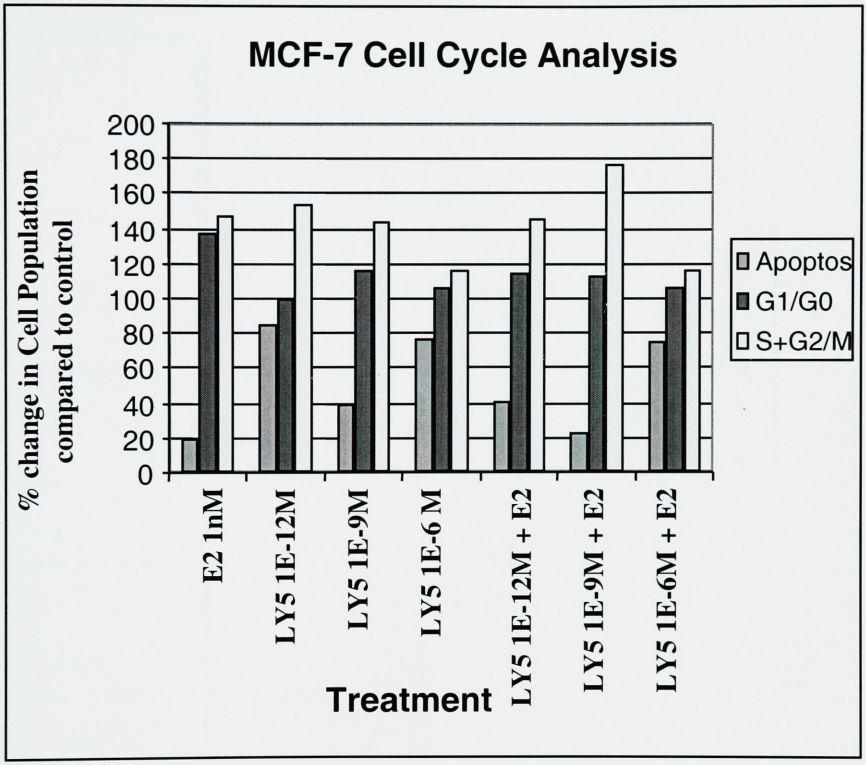
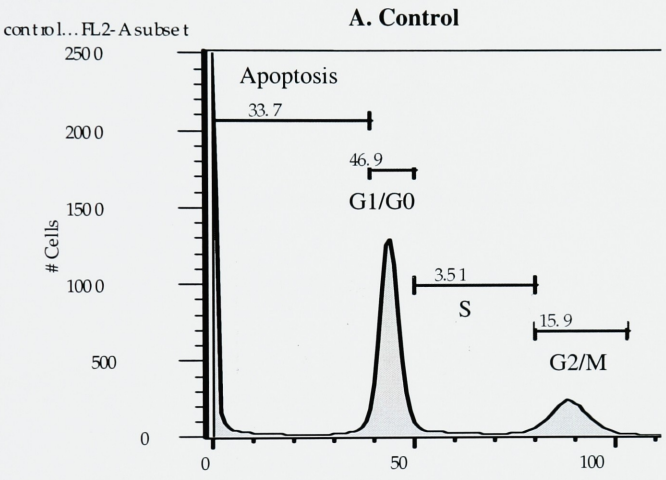
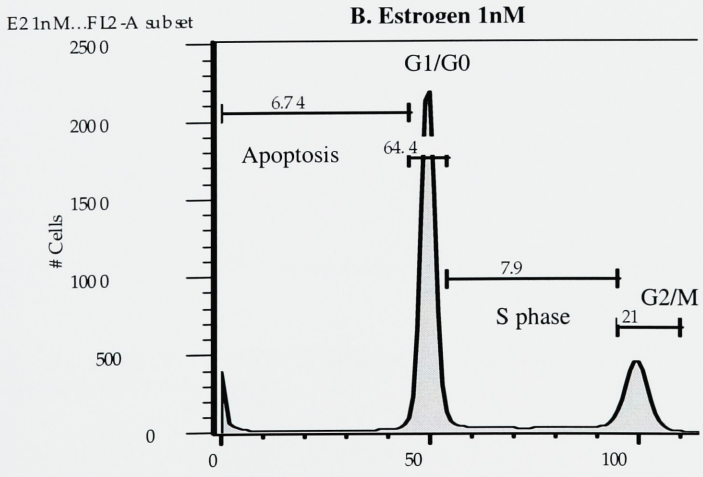


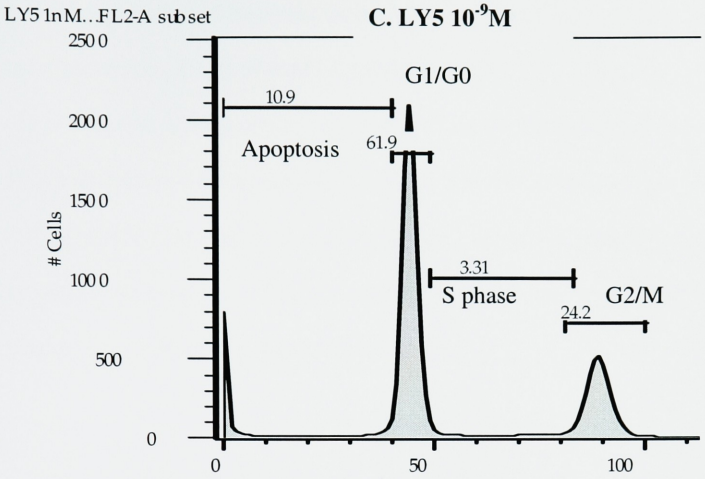

**Figure 4 - MCF-7 Cell Cycle Analysis.** MCF-7 cells cultured in prolonged estrogen deprivation were serum starved for 12 hours and stimulated with estrogen and/or LY5 as shown above.

The addition of LY5 $10^{-12}$M to the estrogen treatment results in a two-fold increase in MCF-7 apoptosis, compared to estrogen alone. The addition of LY5 $10^{-9}$M to the estrogen treatment results in no significant change in the level of apoptosis, however,

there is a slight increase in the S and G2/M phases compared to estrogen alone. Estrogen in combination with LY5 $10^{-6}$M resulted in a three-fold increase in the percentage of cells undergoing apoptosis and a 20% decrease in the S and G2/M phases (similar to LY5 $10^{-6}$M alone) when compared to estrogen alone. The results are shown in Figure 4.





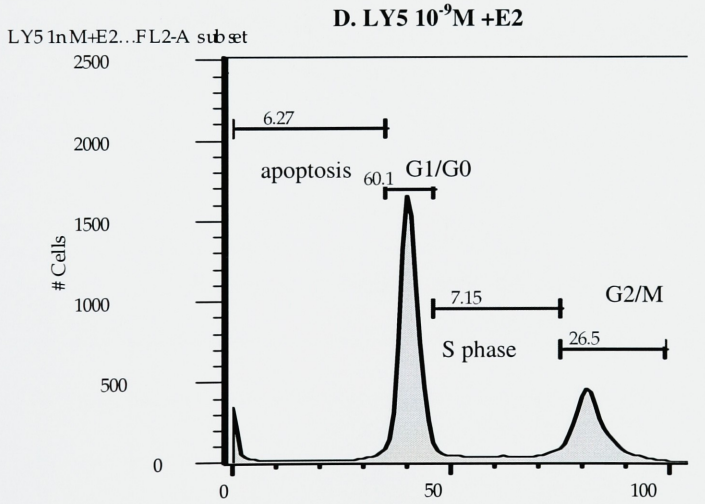
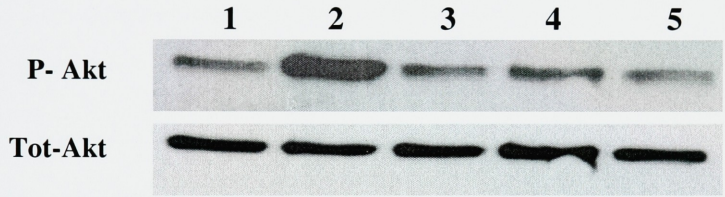




**Figure 5 - MCF-7 Cell Cycle Analysis**. Stimulation with A. control, B. 1nM estrogen C. LY5 $10^{-9}$M and D. LY5 $10^{-9}$M + E1nM.

### 1.4 EA.hy926 Rapid Stimulation with Estrogen and LY5

#### 1.4.1 Induction of Akt phosphorylation by LY5

In order to evaluate the effect of LY5 on the phosphorylation of Akt in EA.hy926 ECs, cultured cells were stimulated with LY5 and or estrogen. Stimulation of EA.hy926 cells with LY5 resulted in a slightly higher level of phosphorylated-Akt (p-Akt) higher compared to estrogen. The combination of LY5 and estrogen resulted in an actual decrease in the level of p-Akt as shown below.




1. Control
2. FBS 20%
3. E2 30nM
4. LY5 $10^{-12}$M
5. LY5 $10^{-12}$M + E2

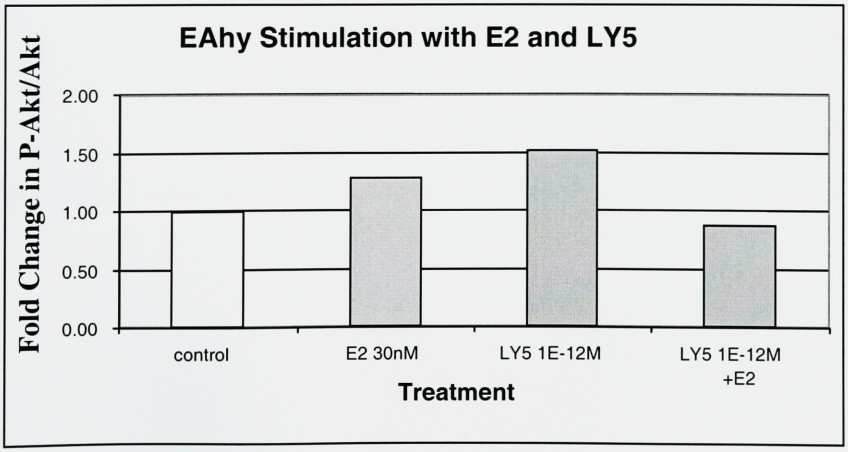
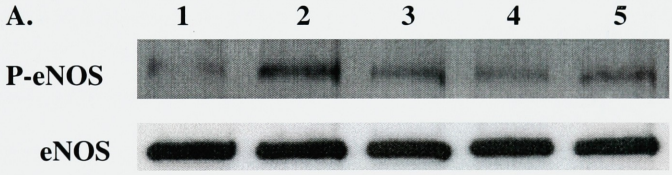


**Figure 6 – Phosphorylation of Akt by LY5 and Estrogen in Endothelial Cells.** Confluent monolayers of EA.hy.926 cells underwent estrogen deprivation for 48 hours, followed by a 12 hour starvation period. The cells were subsequently incubated with LY5 and/or estrogen as shown above for 10 minutes at 37°C. Lysates were subjected to SDS-PAGE, transferred to nitrocellulose, probed with anti-pAkt antibody, and reprobed with anti-Akt antibody.

### 1.4.2 Induction of eNOS phosphorylation by LY5

In order to evaluate the effect of LY5 on the phosphorylation of eNOS in EA.hy926 ECs, cultured cells were stimulated with LY5 and or estrogen. Stimulation of EA.hy926 cells with estrogen resulted in a greater level of phosphorylated-eNOS (p-eNOS) compared to LY5. The addition of LY5 to estrogen did not result in a significant change in the level of p-eNOS compared to estrogen alone.




**1. Control**
**2. FBS 20%**
**3. E2 30nM**
**4. LY5 $10^{-12}$M**
**5. LY5 $10^{-12}$M + E2**

**Figure 7 - Phosphorylation of eNOS by LY5 and estrogen in endothelial cells**

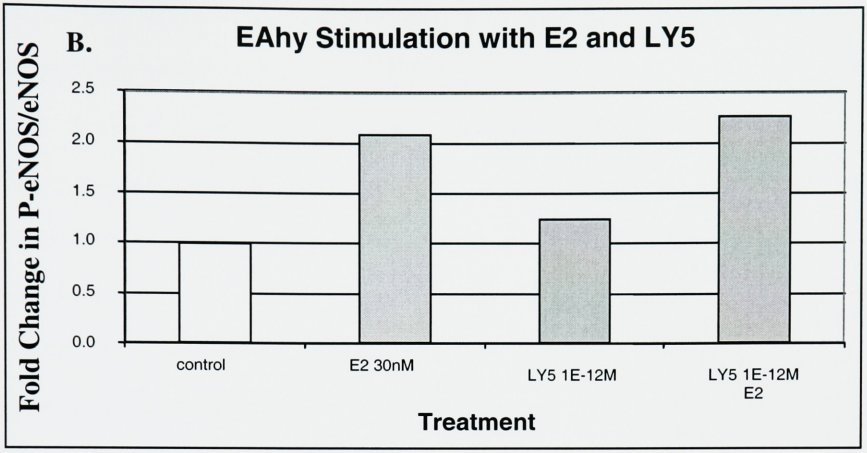



**Figure 7 – Phosphorylation of eNOS by LY5 and estrogen in endothelial cells.** Confluent monolayers of EA.hy.926 cells underwent estrogen deprivation for 48 hours, followed by a 12 hour starvation period. The cells were subsequently incubated with LY5 and/or estrogen as shown in figure A for 10 minutes at 37°C. A. Lysates were subjected to SDS-PAGE, transferred to nitrocellulose, probed with antiphospho eNOS antibody, and reprobed with anti-eNOS antibody.

### 1.5 Summary of Results for LY5

The results of the cell proliferation assay and cell cycle analysis for MCF-7 cells reveal a bell-shaped dose response to LY5, with a $IC_{50}$-value of approximately $10^{-6}$M. At the lowest concentration tested ($10^{-12}$M) it appears that LY5 decreases survival of MCF-7 cells compared to estrogen. At higher concentrations ($10^{-6}$M) LY5 appears to decrease the survival and also decrease the progression of MCF-7 cells through the cell cycle to the S and G2 phases when compared to estrogen. At intermediate concentrations of LY5, however, there is increased survival of MCF-7 cells, but not to the extent seen with estrogen.

The results of the cell proliferation assay for the endothelial cells show increased cell proliferation above control for LY5 between the concentrations $10^{-11}$M and $10^{-7}$M. There is minimal cell proliferation at $10^{-12}$M and $10^{-6}$M, and at higher concentrations ($10^{-}$

$^{5}$M) there is a significant decrease in viable cells, most likely due to a cytotoxic effect on the cells at that concentration.

Stimulation of endothelial cells to evaluate the effects on the rapid phosphorylation of Akt and eNOS, showed that estrogen caused an increase in both p-Akt and p-eNOS as expected since p-Akt directly phosphorylates and activates eNOS (189). Stimulation with LY5 $10^{-12}$M, however, resulted in an increase in p-Akt but not a significant increase in the levels of p-eNOS, suggesting that LY5 may cause downregulation of eNOS, independent of Akt activation, for example through inhibition of the MAP kinase pathway of eNOS activation.

At a concentration of $10^{-6}$M, LY5 exhibits an overall favorable profile with respect to its effects on the breast cancer cell line. LY5 decreased MCF-7 cell proliferation, decreased cell survival, decreased progression to the S and G2/M phases of the cell cycle. Additionally, these effects were not reversible in the presence of estrogen. Although there was an overall favorable effect on endothelial cell proliferation, we did not observe a significant increase in p-eNOS activity after treatment with LY5.

## 2.0 Results for compound LY7

### 2.1 MCF-7 Cell Proliferation Assay for LY7

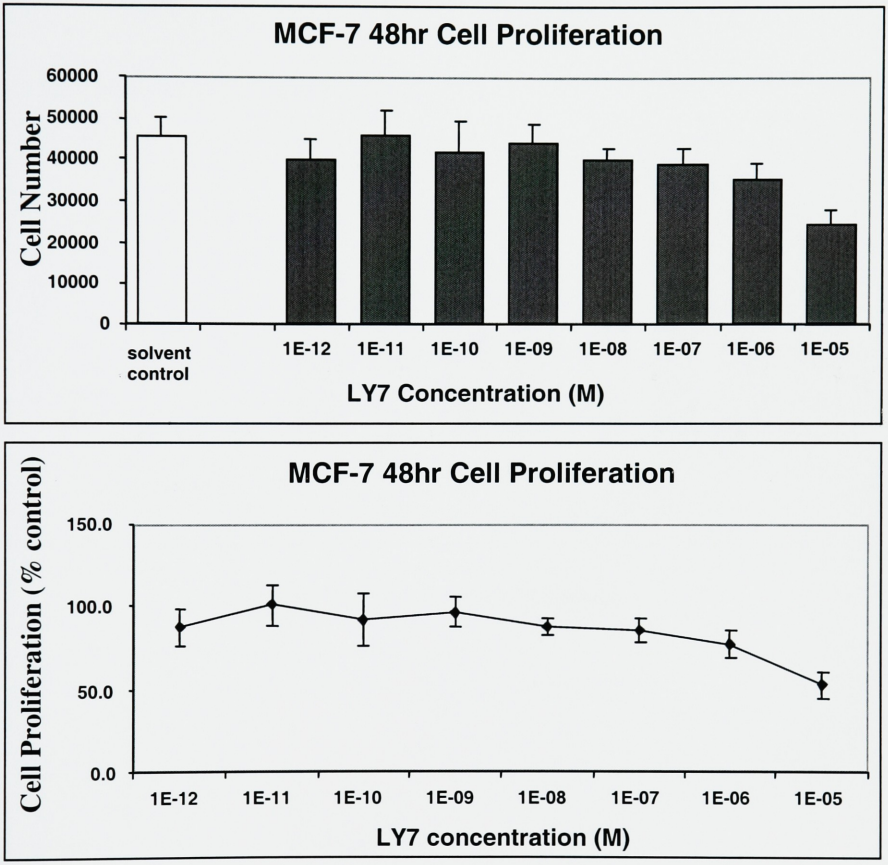



**Figure 8 - Cell proliferation Assay with MCF-7 breast cancer cells.** Multi-well dishes plated in triplicate, 8000 cells/well. 12 hour starvation period before stimulation with LY 7 at the concentrations indicated above. The plates were read 48 hours after stimulation.

Treatment of MCF-7 cells with LY7 for 48hrs showed an overall inhibitory effect on cell proliferation. There was a dose dependent inhibitory effect on cell proliferation, with a statistically significant reduction in cell numbers starting at a concentration of $10^{-}$

$^{8}$M. Cell numbers were approximately in the same range as the control treatment for LY7 at $10^{-12}$M and dropped off by 50% at LY7 $10^{-5}$M, suggesting an overall anti-estrogenic effect for LY7 on MCF-7 cells in the range of concentrations examined.

**2.2 EA.hy926 Cell Proliferation Assay for LY7**

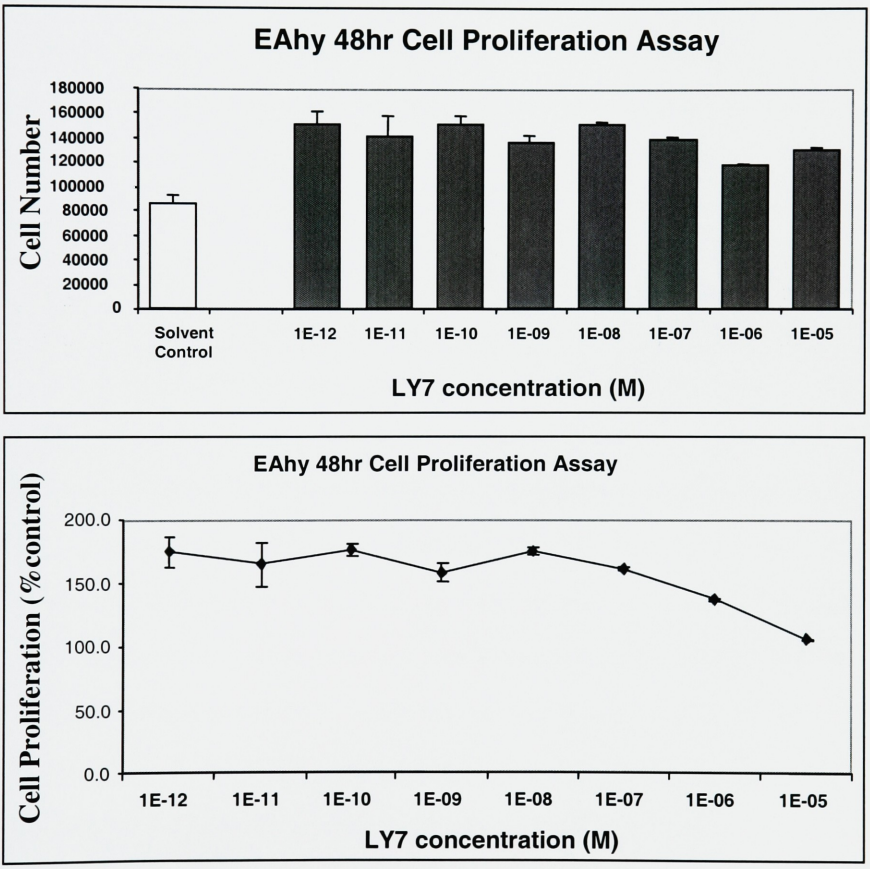



**Figure 9 - Cell proliferation Assay with EA.hy926 endothelial cells.** Multi-well dishes plated in triplicate, 8000 cells/well. The cells were estrogen deprived for 48 hours, followed by a 12 hour starvation period before stimulation with LY 7 at the concentrations indicated above. The plates were read 48 hours after stimulation.

Treatment of endothelial cells with LY7 resulted in an overall increase in cell proliferation when compared to vehicle treatment. At $10^{-12}$M there was a 70% increase in cell numbers compared to control, however, increasing concentrations of LY7 resulted in a subsequent decrease in a cell proliferation. At $10^{-5}$M cell proliferation was at the level of that observed with the control treatment. These results suggest that at low concentrations LY7 exerts an overall estrogenic effect on endothelial cell proliferation, but at increasing concentrations the kinetics of LY7 shift to an anti-estrogenic profile.

**2.3 MCF-7 Cell Cycle Analysis for LY7**

The results of cell cycle analysis performed on MCF-7 cells stimulated with estrogen and or LY7 are shown in Figure 10. At $10^{-12}$M LY7 produced a four-fold increase in the population of apoptotic cells, in addition to a 40% decrease in the S andG2/M phases, compared to estrogen. This suggests that at this concentration LY7 reduces breast cancer cell progression to the S and G2/M phases and also reduces survival as evident by the increase in apoptosis. At higher concentrations, LY7 $10^{-6}$M caused a three-fold increase in apoptosis, and a 15% decrease in the S and G2/M phases when compared to the estrogen treatment. The addition of LY7 $10^{-12}$M or LY7 $10^{-6}$M to the estrogen treatment resulted in a two fold increase in apoptosis, but no significant changes in the S and G2/M phases when compared to estrogen treatment alone, suggesting that LY7 partially antagonizes the survival benefit of estrogen on MCF-7 cells, but does not significantly antagonize the positive effect of estrogen on cell cycle progression.

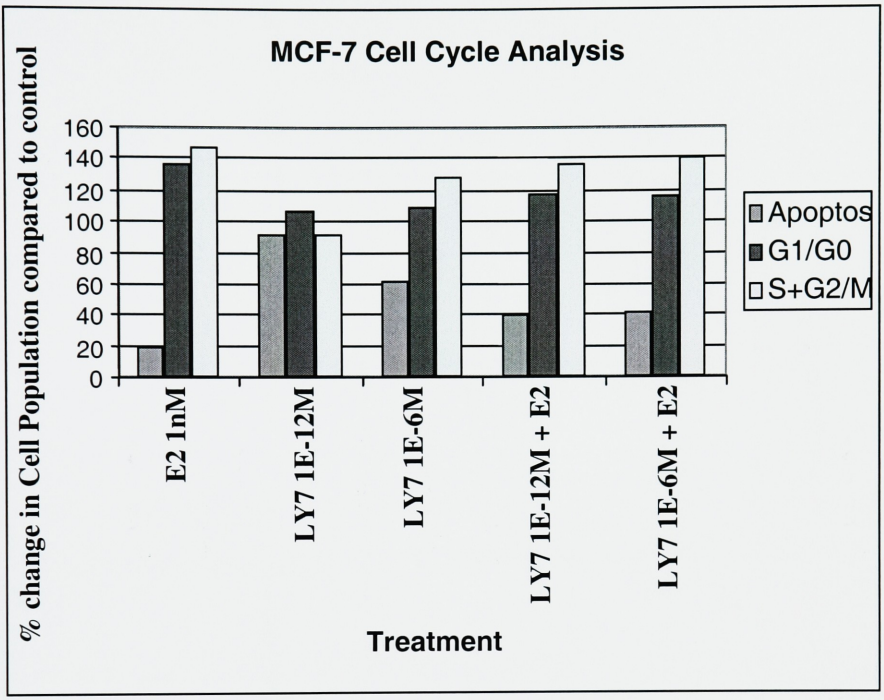
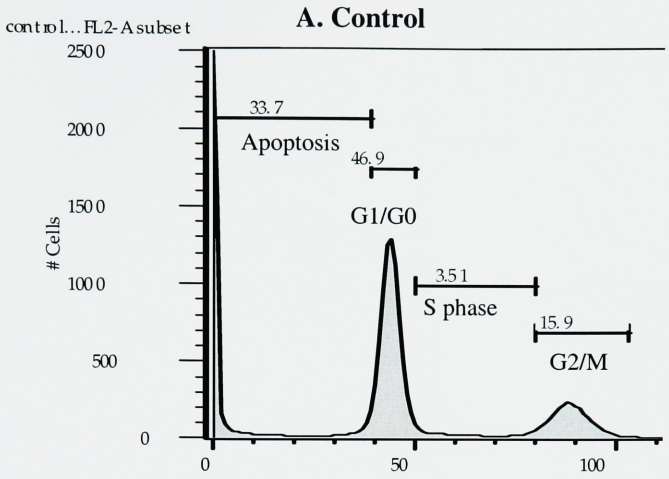
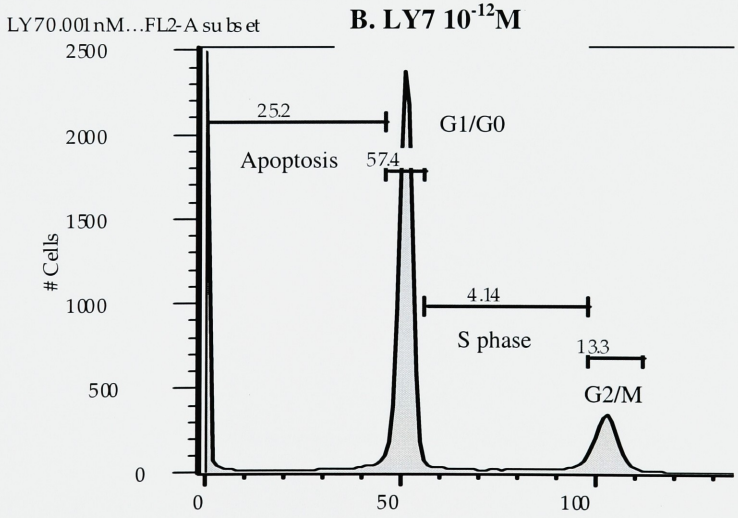



**Figure 10 - MCF-7 Cell Cycle Analysis.** MCF-7 cells cultured in prolonged estrogen deprivation were serum starved for 12 hours and stimulated with estrogen and/or LY7 as shown above.


control...FL2-A subset
A. Control
2500
2000
1500
1000
500
0
# Cells
33.7
Apoptosis
46.9
G1/G0
3.51
S phase
15.9
G2/M
0
50
100


LY70.001nM...FL2-A subset
B. LY7 10-12M
2500
2000
1500
1000
500
0
# Cells
25.2
Apoptosis
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4.14
S phase
13.3
G2/M
0
50
100

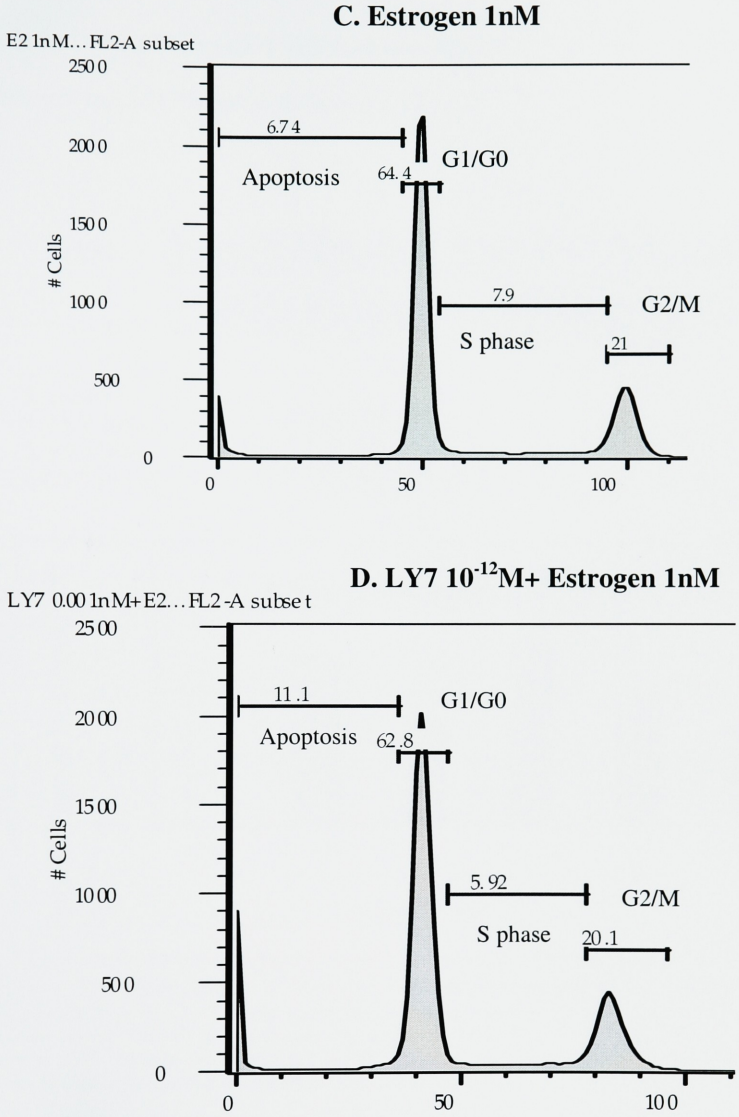
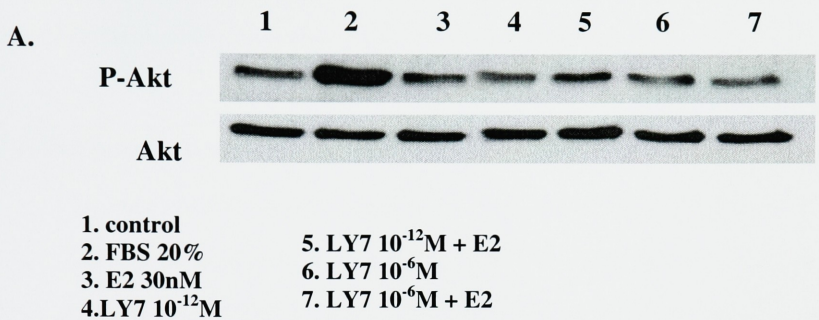



**Figure 11 - MCF-7 Cell Cycle Analysis** Stimulation with A. control, B.LY7 $10^{-12}$M C. 1nM estrogen and D. LY7 $10^{-12}$M + E1nM.

## 2.4 EA.hy926 Rapid Stimulation with Estrogen and LY7

### 2.4.1 Induction of Akt Phosphorylation by LY7



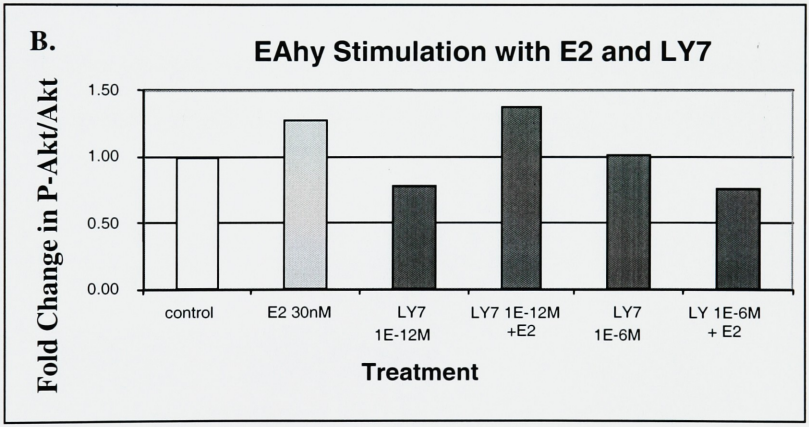




**Figure 12 – Phosphorylation of Akt by LY7 and estrogen in endothelial cells.** Confluent monolayers of EA.hy.926 cells underwent estrogen deprivation for 48 hours, followed by a 12 hour starvation period. The cells were subsequently incubated with LY7 and/or estrogen as shown in A. for 10 minutes at 37°C. Lysates were subjected to SDS-PAGE, transferred to nitrocellulose, probed with antiphospho Akt antibody, and reprobed with anti-Akt antibody.

In order to evaluate the effect of LY7 on the phosphorylation of Akt in EA.hy926 ECs, cultured cells were stimulated with LY7 and/or estrogen. Treatment of EA.hy926 cells with LY7 at $10^{-6}$M and $10^{-12}$M resulted in no significant increase in phosphorylated-Akt (p-Akt) compared to estrogen. The addition of LY7 $10^{-12}$M to estrogen did not result

in a significant change in the level of p-Akt, however, when $10^{-6}$M of LY7 was added to the estrogen treatment there was a 40% reduction in the level of p-Akt, suggesting that at this concentration LY7 antagonizes the positive effect of estrogen on the induction of Akt phosphorylation, and subsequently phosphorylation and activation of eNOS.

### 2.4.2 Induction of eNOS Phosphorylation by LY7

In order to evaluate the effect of LY7 on the phosphorylation of eNOS in EA.hy926 ECs, cultured cells were stimulated with LY7 and or estrogen. Incubation of EA.hy926 cells with LY7 at either concentration did not result in a significant increase in the level of phosphorylated-eNOS (p-eNOS) compared to control. The addition of LY7 $10^{-6}$M or $10^{-12}$M to estrogen resulted in a 35% and 25% decrease respectively in the level of p-eNOS compared to the estrogen treatment alone.

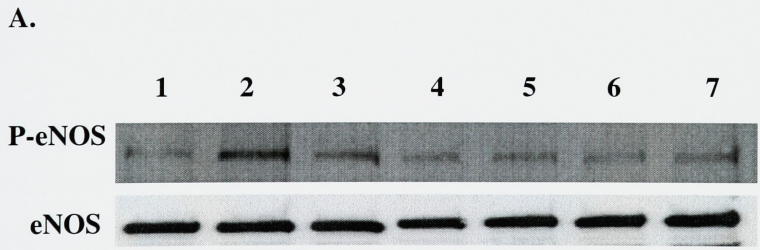



**Figure 13 – Phosphorylation of eNOS by LY7 and estrogen in endothelial cells.**

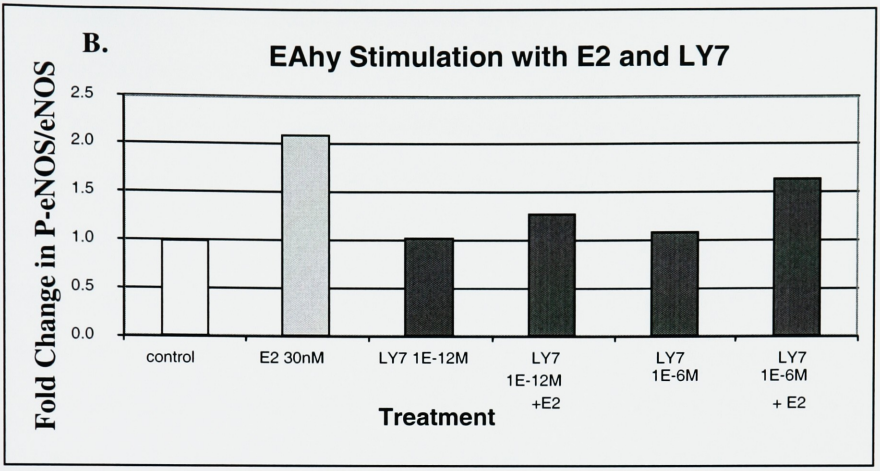



**Figure 13 – Phosphorylation of eNOS by LY7 and estrogen in endothelial cells.** Confluent monolayers of EA.hy.926 cells underwent estrogen deprivation for 48 hours, followed by a 12 hour starvation period. The cells were subsequently incubated with LY7 and/or estrogen as shown in figure A for 10 minutes at 37°C. A. Lysates were subjected to SDS-PAGE, transferred to nitrocellulose, probed with antiphospho eNOS antibody, and reprobed with anti-eNOS antibody. B. Densitometric analysis of p-eNOS/eNOS

### 2.5 Summary of Results for LY7

The results of the cell proliferation assay for the EA.hy926 cells show that LY7 stimulates endothelial cell proliferation. This effect on cell proliferation is more pronounced at the lower concentrations examined and progressively diminishes as the concentration increases. It appears that there is a differential effect of low or high dosages of the compound, with the lower doses causing an increase in endothelial cell proliferation. Higher doses, however, are associated with a negative effect on cell proliferation. This suggests that there may be an optimal concentration at which LY7 may exert full estrogen-like activity, whereas at higher concentrations it may have partial antiestrogenic effects. A similar differential effect of low or high dosages was reported

by Simoncini et al with the experimental SERM aclobifene on eNOS activity in aortas of estrogen deficient rats (25).

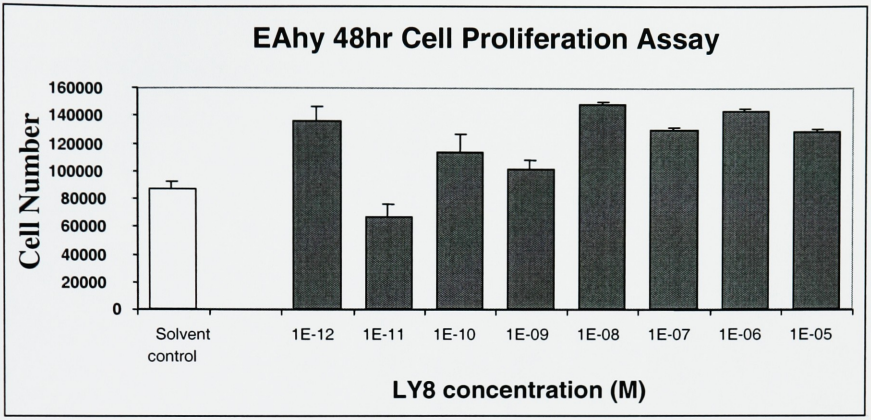
The cell proliferation assay for the MCF-7 cells show that LY7 exhibits an overall inhibitory effect on cell proliferation, which is dose dependent and most pronounced at $10^{-5}$M. Cell cycle analysis of MCF-7 cells showed a significant decrease in the survival of breast cancer cells, but also a decrease in the progression to the S and G2/M phases most pronounced at $10^{-12}$M of LY7.

Stimulation of endothelial cells to evaluate the effects on the rapid phosphorylation of Akt and eNOS showed no significant effect on the levels of p-Akt or p-eNOS for the two concentrations of LY7 examined. Addition of LY7 to estrogen actually resulted in a decrease in the levels of p-eNOS compared to estrogen alone, suggesting an anti-estrogenic effect of LY7 under these conditions.

At a concentration of $10^{-12}$M, LY7 exhibits a favorable profile with respect to its effects on the breast cancer cell line. LY7 decreased MCF-7 cell proliferation, decreased cell survival, and decreased progression to the S and G2/M phases of the cell cycle. Additionally, these effects were not reversible in the presence of estrogen. Although there was an overall favorable effect on endothelial cell proliferation, we did not observe a significant increase in p-eNOS activity after treatment with LY7.

## 3.0 Results for compound LY8

### 3.1 EA.hy926 Cell proliferation Assay



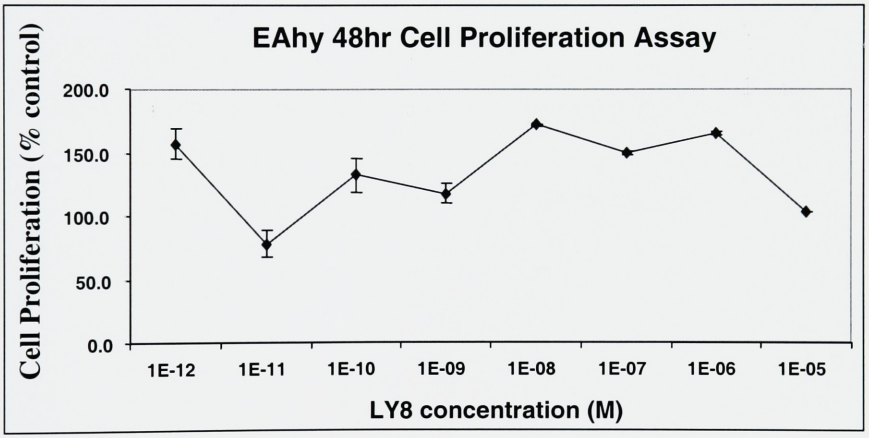




**Figure 15 - Cell proliferation Assay with EA.hy926 endothelial cells.** Multi-well dishes plated in triplicate, 8000 cells/well. The cells were estrogen deprived for 48 hours, followed by a 12 hour starvation period before stimulation with LY 8 at the concentrations indicated above. The plates were read 48 hours after stimulation.

Treatment of endothelial cells with LY8 for 48hrs showed an overall increase in cell proliferation above that seen with the control treatment. The response appears to be

dose dependent, with a peak in cell proliferation seen at LY8 $10^{-8}$M. Further increase in LY8 concentration appears to exert an inhibitory effect on cell proliferation.

### 3.2 MCF-7 Cell Proliferation Assay

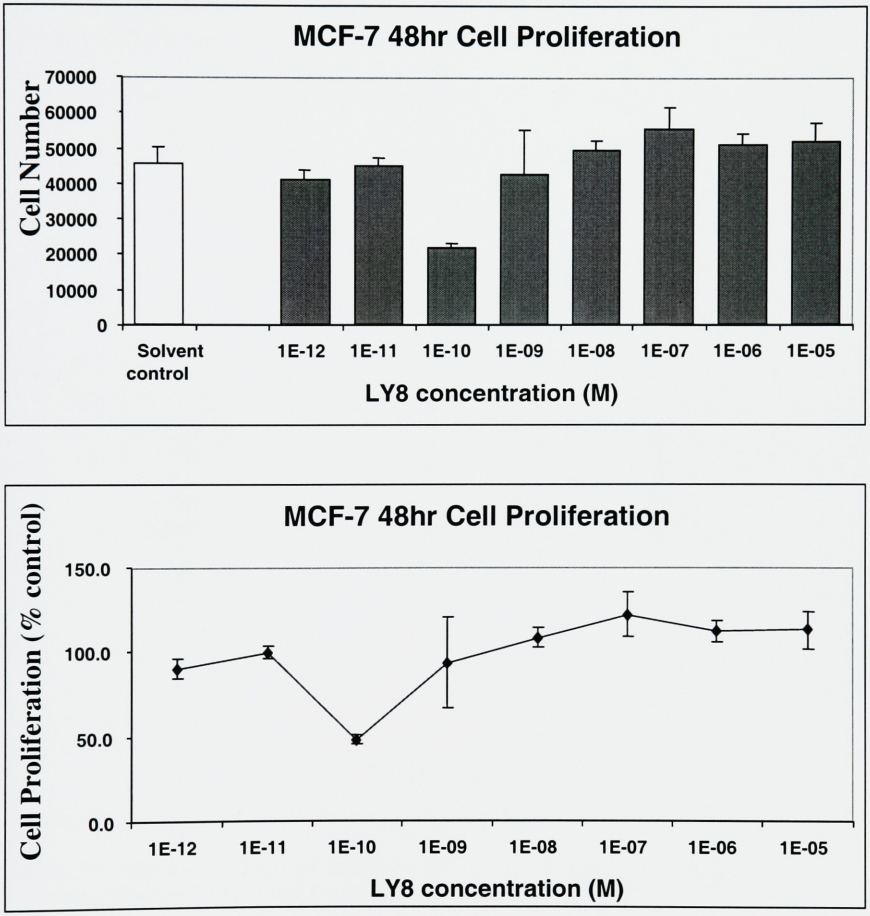



**Figure 16 - Cell proliferation Assay with MCF-7 breast cancer cells.** Multi-well dishes plated in triplicate, 8000 cells/well. There was a 12 hour starvation period before stimulation with LY 8 at the concentrations indicated above. The plates were read 48 hours after stimulation.

Stimulation of MCF-7 cells with LY8 resulted in lower levels of cell proliferation compared to control for concentrations below $10^{-9}$M. There was a slight increase in cell numbers compared to control for concentrations of LY8 greater than $10^{-9}$M.

### 3.3 MCF-7 Cell Cycle Analysis

Cell cycle analysis was performed on MCF-7 cells stimulated with estrogen and or LY8. At LY8 $10^{-12}$M there was a four-fold increase in the level of apoptosis, and there was a 20% decrease in the population of cells found in the S and G2/M phases,

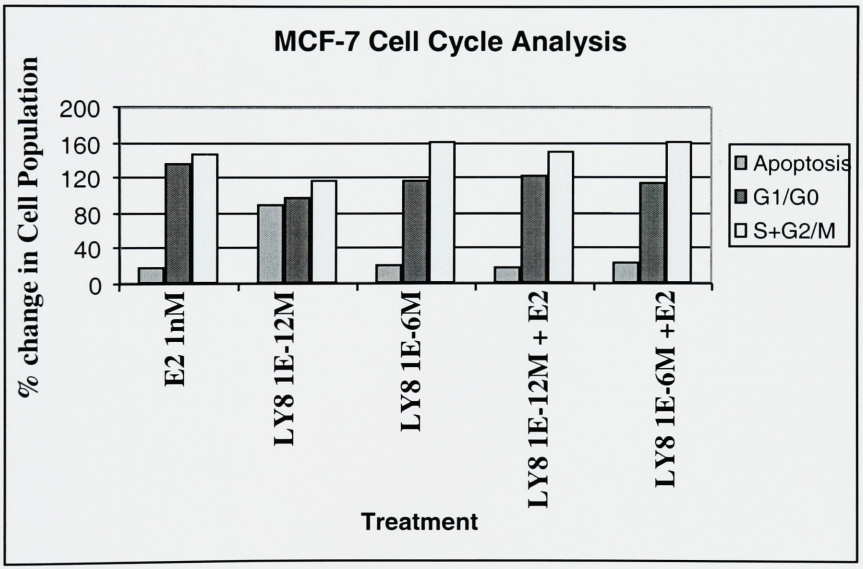
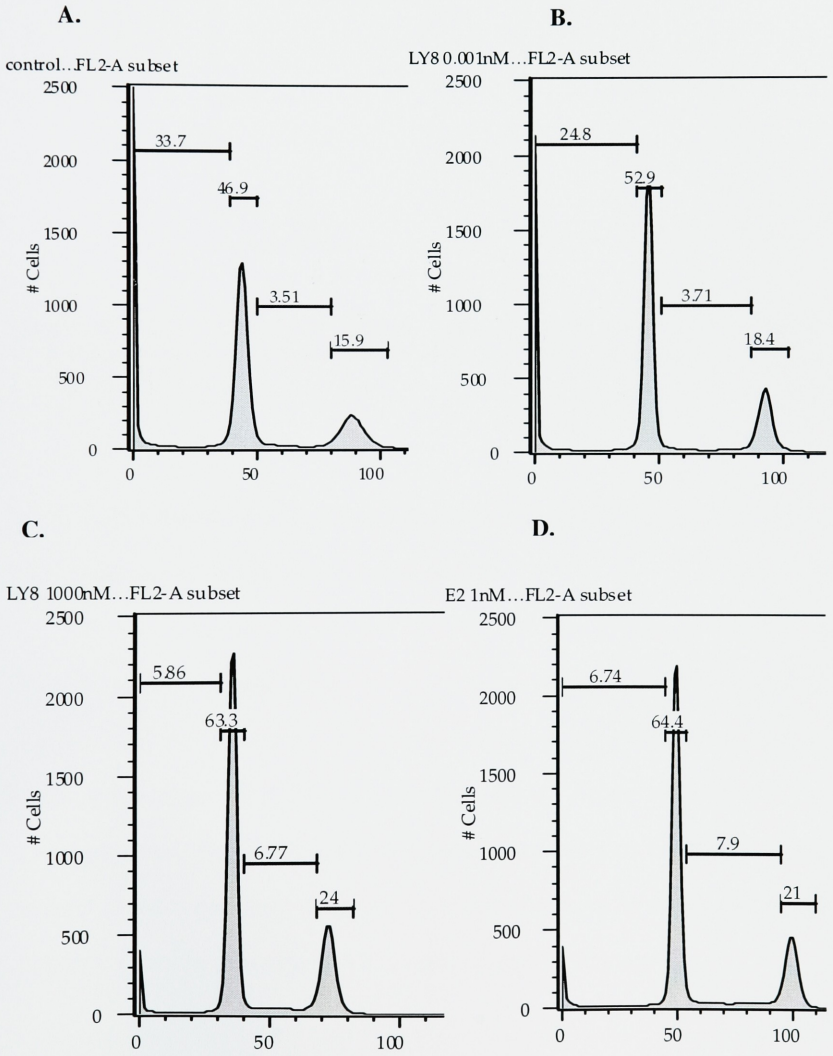



**Figure 17 - MCF-7 Cell Cycle Analysis.** MCF-7 cells cultured in prolonged estrogen deprivation were serum starved for 12 hours and stimulated with estrogen and/or LY8 as shown above.

when compared to estrogen. Treatment with $10^{-6}$M of LY8 did not cause a significant change in the distribution of cells undergoing apoptosis or progressing to the S and G2M phases. At this concentration ($10^{-6}$M) it is possible that LY8 exerts an effect on MCF-7

cells that is similar to estrogen. Although MCF-7 cell treatment with LY8 alone appears to exert an anti-estrogenic effect at $10^{-12}$M, the addition of estrogen to the LY8 $10^{-12}$M treatment seems to override this effect. One possible mechanism for this is that estrogen has a higher affinity for the estrogen receptor, displacing LY8 and diminishing its effect.



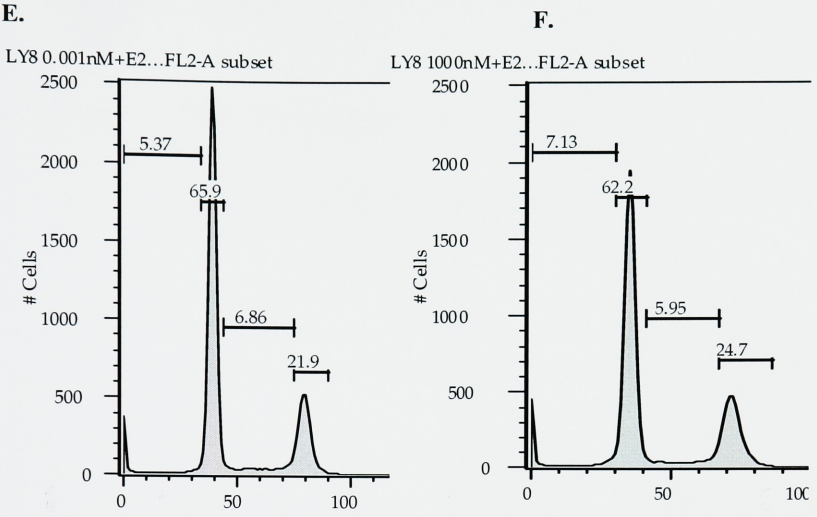


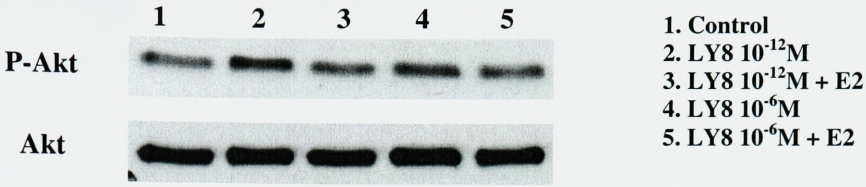

**Figure 18 - MCF-7 Cell Cycle Analysis** Stimulation with A. control, B.LY8 $10^{-12}$M C. LY8 $10^{-6}$M, D estrogen $10^{-9}$M, E. LY8 $10^{-12}$M + Estrogen $10^{-9}$M, F. LY8 $10^{-6}$M + Estrogen $10^{-9}$M

### 3.4 EA.hy926 Rapid Stimulation with Estrogen and LY8

#### 3.4.1 Induction of Akt phosphorylation by LY8

In order to evaluate the effect of LY8 on the phosphorylation of Akt in EA.hy926 ECs, cultured cells were stimulated with LY8 and/or estrogen. Stimulation of EA.hy926 cells with LY8 at $10^{-6}$M resulted in comparable levels of phosphorylated-Akt (p-Akt) compared to estrogen. LY8 $10^{-12}$M resulted in a slightly higher level of p-Akt compared to estrogen alone. The addition of LY8 to the estrogen treatment resulted in a decrease in the level of p-Akt compared to the estrogen treatment alone, suggesting an antagonistic effect of LY8 on the induction of Akt phosphorylation by estrogen.

A.



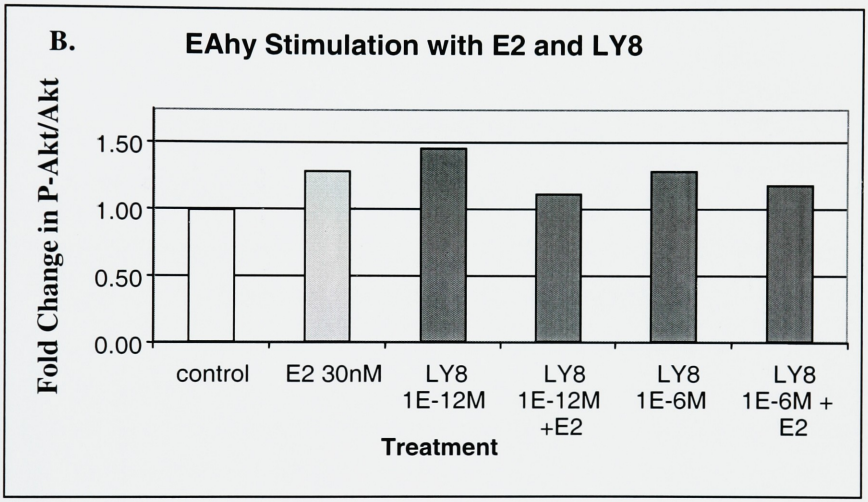




**Figure 19 – Phosphorylation of Akt by LY8 and estrogen in endothelial cells.** Confluent monolayers of EA.hy.926 cells underwent estrogen deprivation for 48 hours, followed by a 12 hour starvation period. The cells were subsequently incubated with LY8 and/or estrogen as shown in A. for 10 minutes at 37°C. A. Lysates were subjected to SDS-PAGE, transferred to nitrocellulose, probed with antiphospho Akt antibody, and reprobed with anti-Akt antibody. B. Densitometric analysis of pAkt/Akt levels after stimulation with estrogen and/or LY8

### 3.4.2 Induction of eNOS Phosphorylation by LY8

In order to evaluate the effect of LY8 on the phosphorylation of eNOS in EA.hy926 ECs, cultured cells were stimulated with LY8 and/or estrogen. Incubation of EA.hy926 cells with LY8 $10^{-12}$M did not result in a significant increase in the level of

phosphorylated-eNOS (p-eNOS) compared to control. The addition of LY8 10-12M to estrogen resulted in an increase in the level of p-eNOS compared to estrogen alone, suggesting a synergistic effect of LY8 with estrogen. At LY8 $10^{-6}$M actually resulted in a 50% decrease in p-eNOS levels, suggesting an anti-estrogenic effect at this concentration. At $10^{-6}$M LY8 also decreased the ability of estrogen to induce eNOS phosphorylation, reducing levels of p-eNOS by 30%.

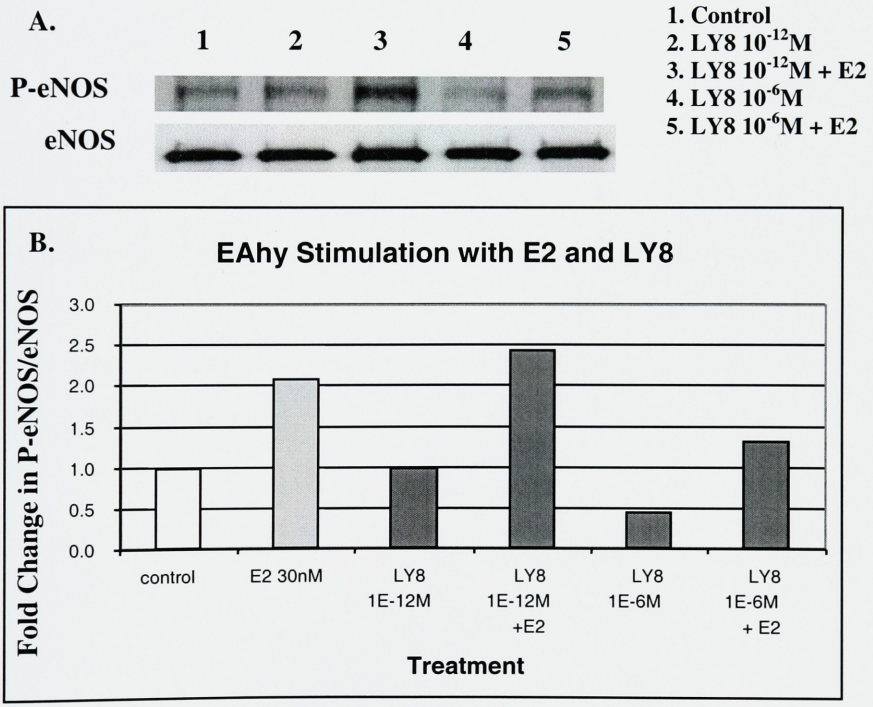



**Figure 20 – Phosphorylation of eNOS by LY8 and estrogen in endothelial cells.** Confluent monolayers of EA.hy.926 cells underwent estrogen deprivation for 48 hours, followed by a 12 hour starvation period. The cells were subsequently incubated with LY8 and/or estrogen as shown in figure A for 10 minutes at 37°C. A. Lysates were subjected to SDS-PAGE, transferred to nitrocellulose, probed with antiphospho eNOS antibody, and reprobed with anti-eNOS antibody. B. Densitometric analysis of p-eNOS/eNOS after stimulation with estrogen and/or LY8.

### 3.5 Summary of Results for LY8

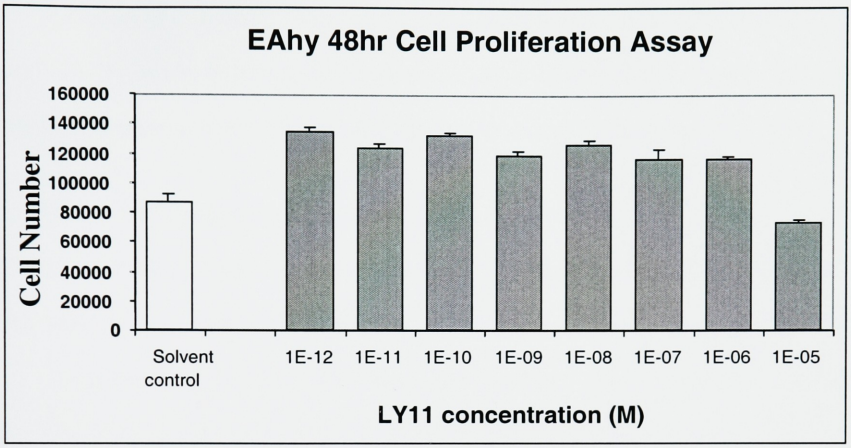
The results of the cell proliferation assay for the EA.hy926 cells show that LY8 stimulates endothelial cell proliferation. There is peak cell proliferation at 10-8M, with lower levels of proliferation at higher and lower concentrations of LY8.

MCF-7 cell showed a dose dependent increase in cell proliferation in response to LY8, with peak proliferation occurring at levels just above those seen in the control group. Cell cycle analysis of MCF-7 cells showed a significant decrease in the survival of breast cancer cells, but also a decrease in the progression to the S and G2/M phases when stimulated with $10^{-12}$M of LY8. This effect MCF-7 cells disappeared in the presence of estrogen.

Stimulation of endothelial cells to evaluate the effects on the rapid phosphorylation of Akt and eNOS showed that LY8 alone induced Akt phosphorylation at levels similar to those seen with estrogen. However, LY8 resulted in a decrease of p-eNOS levels compared to estrogen alone, and in combination with estrogen resulted in lower levels of e-NOS phosphorylation.

## 4.0 Results for compound LY11

### 4.1 EA.hy926 Cell proliferation Assay



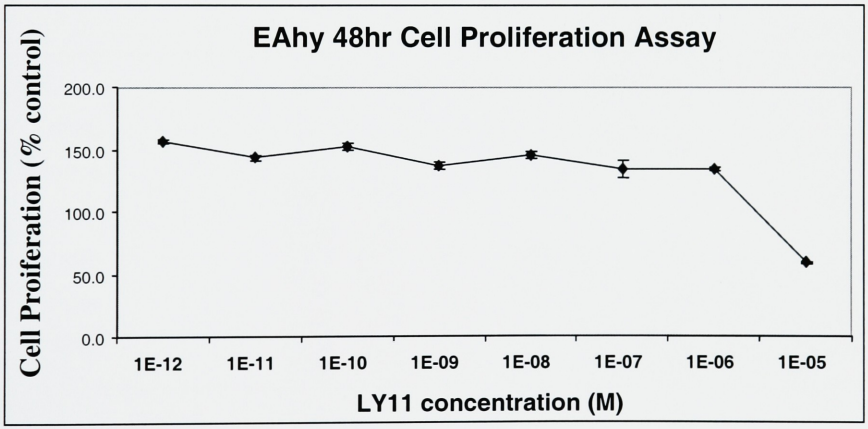



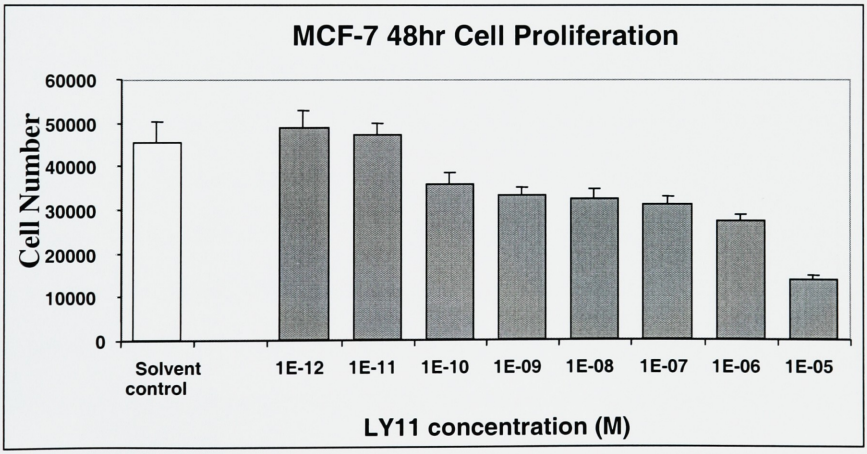

**Figure 21 - Cell proliferation Assay with EA.hy926 endothelial cells.** Multi-well dishes plated in triplicate, 8000 cells/well. The cells were estrogen deprived for 48 hours, followed by a 12 hour starvation period before stimulation with LY 11 at the concentrations indicated above. The plates were read 48 hours after stimulation.

Treatment of endothelial cells with LY11 resulted in increased proliferation for the concentration range between $10^{-12}$M and $10^{-6}$M. There was a 60% increase in cell

proliferation for LY11 at $10^{-12}$ compared to control treatment. The degree of cell proliferation decreased with increasing dose of LY11, but was still 30% higher for LY11 $10^{-6}$M compared to control. There was a precipitous drop in cell proliferation, however, for LY$10^{-5}$M at a level 50% lower compared to the control treatment. This may represent the concentration at which LY11 exerts an overall toxic effect on endothelial cells.

### 4.2 MCF-7 Cell Proliferation Assay for LY11

Treatment of MCF-7 cells with LY11 for 48hrs resulted in an overall inhibitory effect on cell proliferation. Cell numbers were at similar levels as control for concentrations of LY11 smaller than $10^{-11}$M. At higher concentrations there was a dose dependent decrease in cell proliferation, with a 70% reduction for LY11 $10^{-5}$M compared to control.



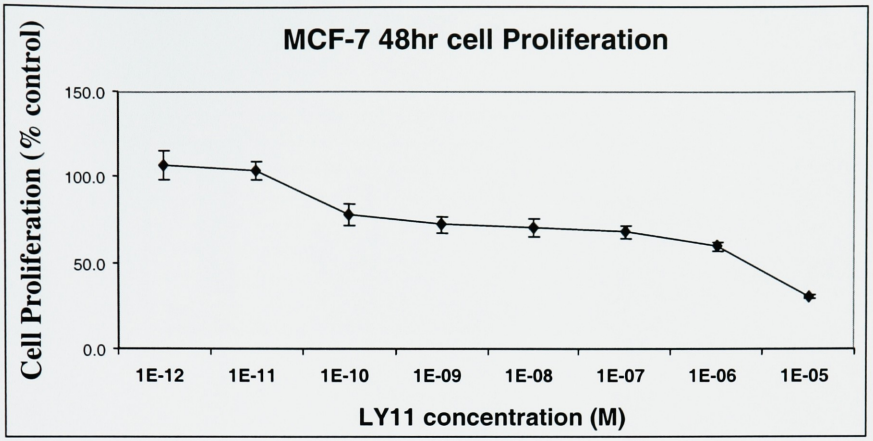



**Figure 22 - Cell proliferation Assay with MCF-7 breast cancer cells.** Multi-well dishes plated in triplicate, 8000 cells/well. There was a 12 hour starvation period before stimulation with LY 11 at the concentrations indicated above. The plates were read 48 hours after stimulation.

### 4.3 MCF-7 Cell Cycle Analysis for LY11

Cell cycle analysis was performed on MCF-7 cells stimulated with estrogen and or LY11. Treatment with LY11 $10^{-12}$M resulted in a four-fold increase in the percentage of apoptotic cells, and a 25% decrease in S and G2/M phase populations, compared to estrogen. The addition of LY11 $10^{-12}$M to estrogen resulted in a two-fold increase in apoptosis, but no significant decrease in the population of cells in the S and G2/M phases, suggesting that at this concentration LY11 decreases the survival benefit conferred by estrogen on the breast cancer cells, with no significant change in cell cycle progression. Treatment with LY11 $10^{-6}$M resulted in a five-fold increase in apoptosis and a 30% decrease in cell cycle progression to the S and G2/M phases.

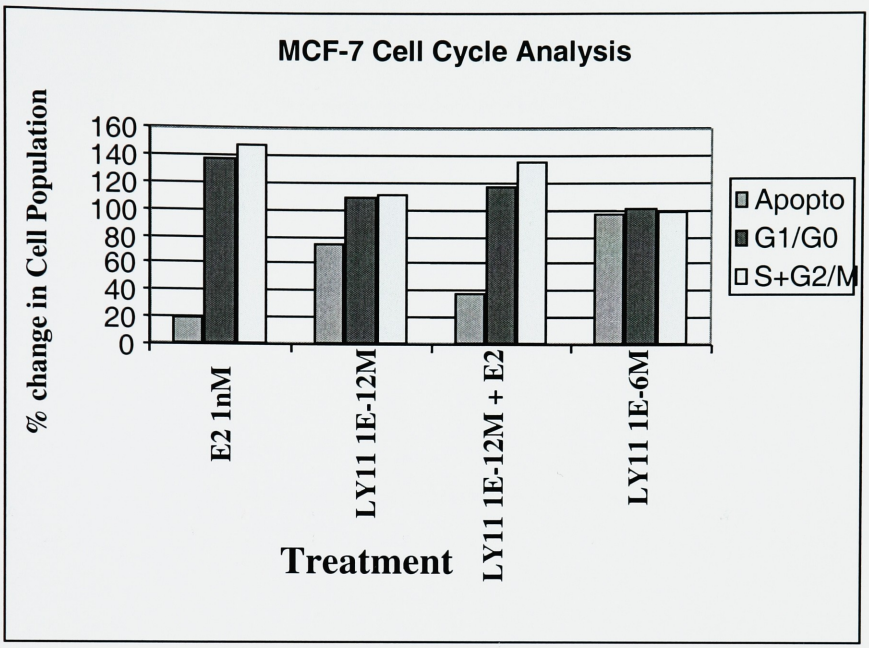
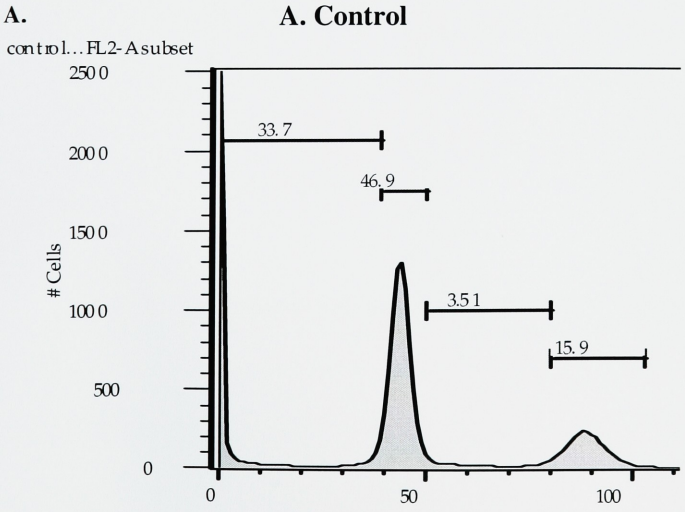
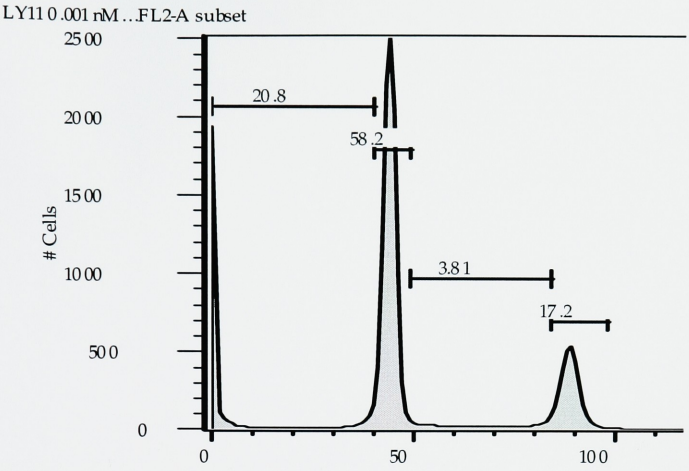



**Figure 23 - MCF-7 Cell Cycle Analysis.** MCF-7 cells cultured in prolonged estrogen deprivation were serum starved for 12 hours and stimulated with estrogen and/or LY11 as shown above.



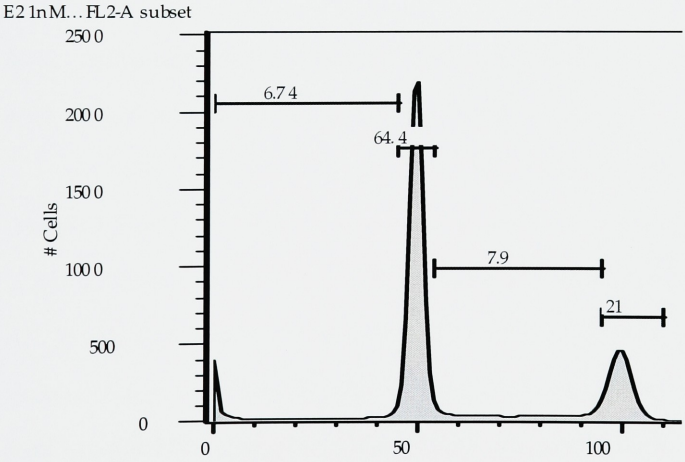
B.

## B. LY11 $10^{-12}$M




C.

## C. Estrogen 1nM



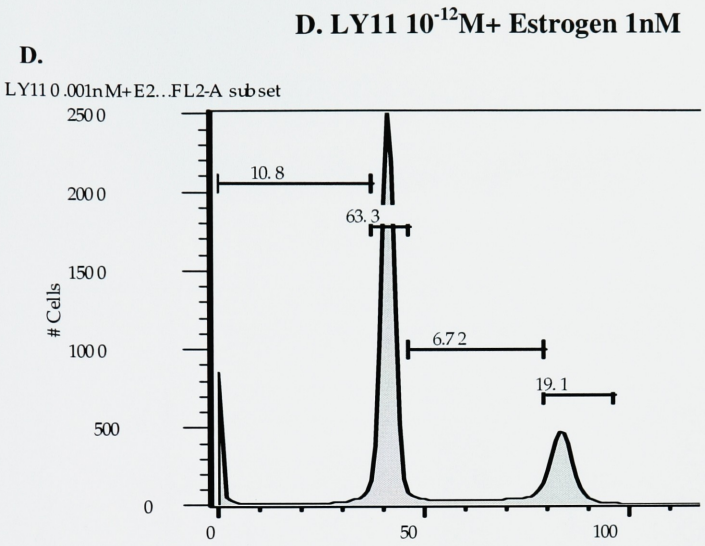



**Figure 24 - MCF-7 Cell Cycle Analysis.** Stimulation with A. control, B.LY11 $10^{-12}$M C. estrogen $10^{-9}$M and D. LY7 $10^{-12}$M + Estrogen $10^{-9}$M

### 4.4 EA.hy926 Rapid Stimulation with Estrogen and LY11

#### 4.4.1 Induction of Akt phosphorylation by LY11

In order to evaluate the effect of LY11 on the phosphorylation of Akt in EA.hy926 ECs, cultured cells were stimulated with LY11 and/or estrogen. Stimulation of EA.hy926 cells with LY11 at $10^{-12}$M resulted in a comparable level of phosphorylated-Akt (p-Akt) compared to estrogen, and LY11 $10^{-6}$M treatment resulted in a 1.6 fold increase of p-Akt compared to control. The addition of LY11 to the estrogen did not result in a significant change in the level of p-Akt compared to estrogen alone.

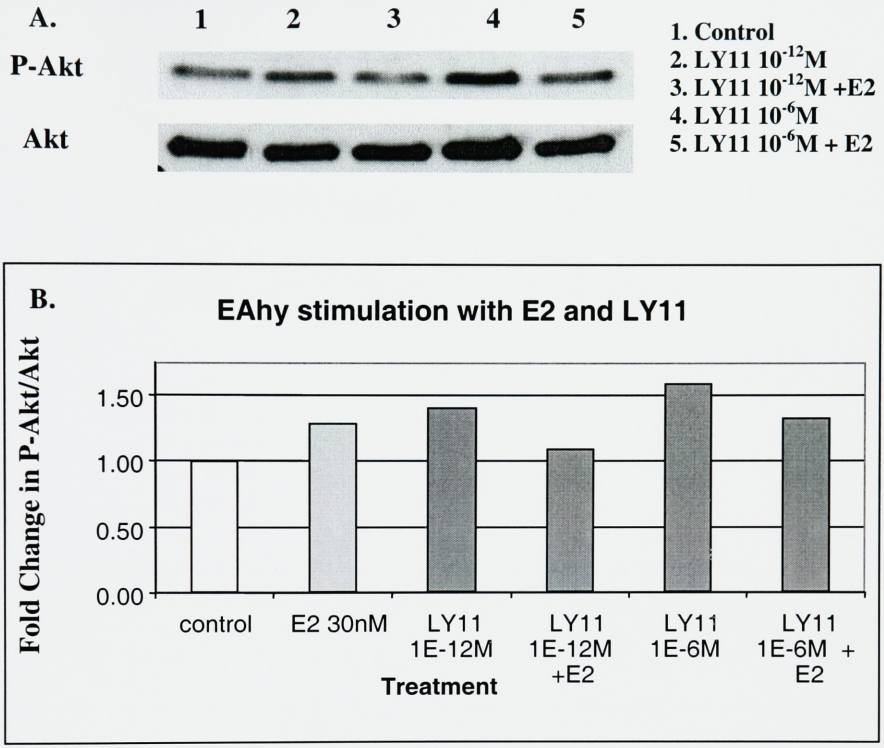
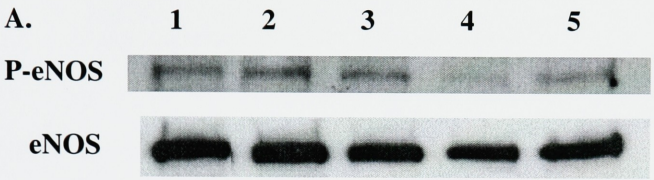



**Figure 25 – Phosphorylation of Akt by LY11 and estrogen in endothelial cells.** Confluent monolayers of EA.hy.926 cells underwent estrogen deprivation for 48 hours, followed by a 12 hour starvation period. The cells were subsequently incubated with LY11 and/or estrogen as shown in A. for 10 minutes at 37°C. A. Lysates were subjected to SDS-PAGE, transferred to nitrocellulose, probed with antiphospho Akt antibody, and reprobed with anti-Akt antibody. B. Densitometric analysis of pAkt/Akt after stimulation with estrogen and LY11.

### 4.4.2 Induction of eNOS phosphorylation by LY11

In order to evaluate the effect of LY11 on the phosphorylation of eNOS in EA.hy926 ECs, cultured cells were stimulated with LY11 and or estrogen. Incubation of EA.hy926 cells with LY11 $10^{-12}$M did not result in a significant increase in the level of phosphorylated-eNOS (p-eNOS) compared to control. LY11 $10^{-6}$M actually resulted in a

significant decrease in p-eNOS. The addition of LY11 $10^{-12}$M or $10^{-6}$M to estrogen resulted in a two-fold decrease in the level of p-eNOS compared to estrogen alone.




1. Control
2. LY11 $10^{-12}$M
3. LY11 $10^{-12}$M +E2
4. LY11 $10^{-6}$M
5. LY11 $10^{-6}$M + E2

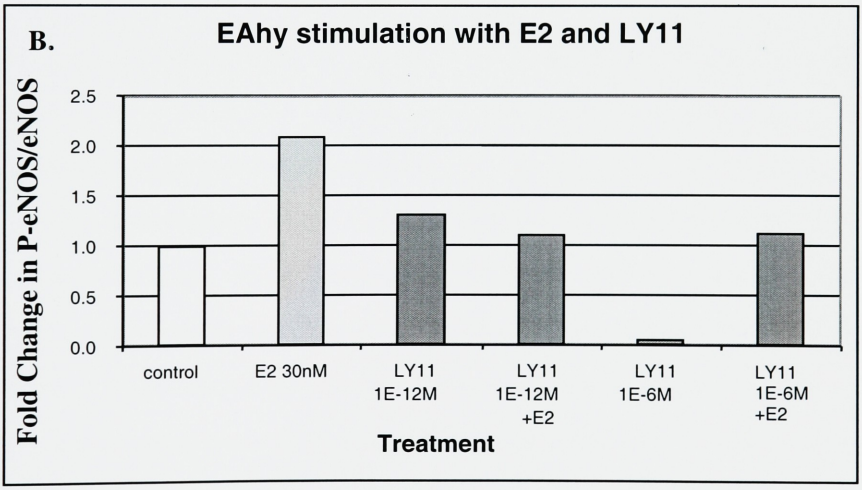



**Figure 26 – Phosphorylation of eNOS by LY11 and Estrogen in Endothelial cells.**
Confluent monolayers of EA.hy.926 cells underwent estrogen deprivation for 48 hours, followed by a 12 hour starvation period. The cells were subsequently incubated with LY11 and/or estrogen as shown in figure A for 10 minutes at 37°C. A. Lysates were subjected to SDS-PAGE, transferred to nitrocellulose, probed with antiphospho eNOS antibody, and reprobed with anti-eNOS antibody. B. Densitometric analysis of p-eNOS/eNOS after stimulation with estrogen and LY11.

**Summary of Results for LY11**

The results of the cell proliferation assay show that LY11 causes an increase in cell proliferation in endothelial cells at concentrations lower than $10^{-6}$M. LY11 caused a dose dependent inhibition of MCF-7 cell proliferation.

The results of the cell cycle analysis show that LY11 caused a decrease in survival and a decrease in cell cycle progression, which was most pronounced at $10^{-6}$M. LY11 was also shown to antagonize the cell survival benefit that estrogen exhibits on MCF-7 cells.

Stimulation of endothelial cells to evaluate the effects on the rapid phosphorylation of Akt and eNOS showed that although LY11 exerted a positive effect on the induction of Akt phosphorylation at levels similar to those seen with estrogen, there was a negative effect on p-eNOS levels compared to estrogen alone. The combination of LY11 with estrogen also resulted in lower levels of e-NOS phosphorylation compared to estrogen alone.

At a concentration of $10^{-6}$M, LY11 exhibits an overall favorable profile with respect to its effects on the breast cancer cell line. LY11 decreased MCF-7 cell proliferation, decreased cell survival, and decreased progression to the S and G2/M phases of the cell cycle better than any of the other compounds examined. Although there was an overall favorable effect on endothelial cell proliferation, we did not observe a significant increase in p-eNOS activity after treatment with LY11.

**Discussion**

Cardiovascular disease is a major concern for postmenopausal women, and estrogen deficiency represents an important factor in the pathophysiology of coronary heart disease. The value of postmenopausal therapy with estrogen or estrogen/progestin in the prevention and treatment of menopause-related symptoms and accelerated bone loss is well established. Observational studies have demonstrated that HRT users have a reduced risk of age-associated diseases or conditions such as osteoporosis, cardiovascular disease, and Alzheimer's disease. Recent randomized controlled trials, however, have found that HRT has actually been associated with serious adverse events on the cardiovascular system in both primary and secondary cardiovascular disease prevention studies. Thus, the use of HRT has remained limited. Additionally, adherence is poor, with the overwhelming majority of women discontinuing treatment because of the fears of cancer of the breast or uterus, unwanted bleeding, and other side effects.

The development of compounds that act as estrogens selectively on the desired tissues has major clinical significance. One of the required goals for an ideal molecule is that the positive effects of estrogens on the cardiovascular system not be lost. SERMs bind with high affinity to the ER but have tissue-specific effects distinct from estradiol. They act as estrogen agonists in some tissues and estrogen antagonist in others. The development of SERMs that selectively interact with specific receptors and specific coactivators and corepressors in specific organ systems offers the possibility of improving the risk/benefit profile relative to HRT and perhaps even extending the use of these compounds to men.

Although approved SERMs such as tamoxifen and raloxifene have potential clinical utility in breast cancer and osteoporosis prevention, widespread use in risk reduction for breast cancer and osteoporosis is limited by an increased risk of adverse events or side effects. Disadvantages of tamoxifen include an increased risk of endometrial cancer and venous thromboembolic disease, as well as hot flashes. Even though raloxifene does not increase the risk of endometrial cancer like tamoxifen, it increases the risk of hot flashes and venous thromboembolic disease, and its cardiovascular and cognitive effects are uncertain.

Ideally, a SERM would have to provide superior efficacy in the treatment and prevention of breast cancer relative to tamoxifen, with an improvement in the toxicity profile. The ideal SERM should also be effective in the prevention and treatment of osteoporosis. Certainly, a property of an ideal SERM would be to provide beneficial effects for the cardiovascular system in women with and without preexisting coronary heart disease. Since molecular and animal research have shown a beneficial effect of estrogen on nerve cells, further desirable effects of the ideal SERM would be to maintain or improve cognitive function and delay or, if possible, prevent the development of dementia. Current SERMs increase or induce hot flashes and therefore cannot be used to treat menopausal symptoms. A property of an ideal SERM would be to provide relief from menopausal symptoms and, certainly, not to exacerbate these symptoms.

The molecular mechanisms for the tissue-specific effects of SERMs involve a unique interplay between the two types of estrogen receptors, ER-$\alpha$ and ER-$\beta$, and a series of coactivator and corepressor proteins. Because of this complexity the effect of

SERMs on the profile of tissue-specific events is still unpredictable. The objective of this study is to identify novel SERMs that have the initial potential to exert the vascular protective effects of estradiol, without exhibiting unfavorable pro-proliferative effects on mammary epithelial cells.

The effects of estrogen on the cardiovascular system are pleiotropic, but many of the beneficial effects are exerted through actions on vascular cells (19,191). Endothelial cells affect the homeostasis of the vessel wall in terms of vasomotor tone, platelet and monocyte adhesion, growth of smooth muscle cells, and extracellular matrix production and thereby provide an antithrombotic and anti-inflammatory barrier for the normal vessel wall. Disruption of the anatomic and functional integrity of the endothelium has been postulated as a mechanism for the initiation of atherosclerosis (192). Experiments by Krasinski et al. and White et al. have suggested that after arterial injury, acceleration of reendothelialization by 17ß-estradiol is associated with attenuation of intimal hyperplasia (61,193). It has also been suggested that increased endothelial cell turnover mediated through accelerated apoptosis induced by TNF-α plays a role in endothelial disruption (70). A study by Alvarez et al. demonstrated a protective effect of 17β-estradiol against TNF-α mediated apoptosis in cultured endothelial cells, suggesting that estradiol may help migrating cells to colonize an injured vessel and aid in reendothelialization.

Accordingly, we chose to use a cell proliferation assay as a screening tool to identify compounds that stimulate endothelial cell proliferation. At the same time we also chose to identify compounds that inhibit or at least do not stimulate MCF-7 breast

cancer cell proliferation, since the importance of estrogen in the regulation of breast cancer cell proliferation has been demonstrated both *in vitro* and *in vivo* (197). The clinical efficacy of oophorectomy and pharmacological agents that inhibit the synthesis or action of estrogen further emphasize the importance of estrogen induced mitogenesis in breast cancer (198).

These initial screening tests allowed us to identify 4 compounds that exhibited both a favorable increase in endothelial cell proliferation and a favorable decrease in breast cancer cell proliferation. The four compounds chosen for further experiments (LY5, LY7, LY8 and LY11) all caused an overall increase in endothelial cell proliferation. LY5, however, caused a rather significant drop in cell numbers at the highest concentration tested ($10^{-5}$M). The fact that there were no viable cells present after 48hrs of exposure to LY5 suggests a toxic effect on endothelial cells at concentrations in the $10^{-5}$M range. (With an anti-estrogenic effect one might expect a more gradual decrease in cell numbers with increasing concentrations of the compound.) This toxic effect, of course, may render the compound unsafe for therapeutic purposes.

The results of the MCF-7 cell proliferation assays revealed favorable profiles for compounds LY7 and LY11. These two compounds did not cause stimulation of MCF-7 proliferation above that observed with control for the concentration range tested, which is a very important property for a SERM to exhibit. LY11 also has a more potent inhibitory effect on the breast cancer cell line compared to LY7. Although both LY5 and LY8 exert an inhibitory effect on MCF-7 proliferation, this effect is not consistent along the entire range of concentrations examined. Unfortunately, the increase in cell proliferation

observed above control from some of the concentrations render these compounds less than ideal as potential SERMs.

In order to further evaluate the effect of the selected potential SERMs on the breast cancer cell line, cell cycle analysis was performed. In order to prevent or treat breast cancer we expect the ideal SERM to cause a decrease in MCF-7 survival and a decrease in progression to the S and G2/M phases of the cell cycle. At both concentrations tested compound LY11 shows the most favorable results, with an increase in the percentage of cancer cells undergoing cell death, and a decrease in the number of cancer cells progressing through the cell cycle. LY11 also inhibits the survival benefit that estrogen has on the breast cancer cell line, and decreases the percentage of cells progressing through the cell cycle. LY7 exhibits a profile similar to LY11, but is less effective at slowing cell cycle progression at the higher of the two concentrations tested. Although both LY5 and LY8 are able to increase the percentage of cancer cells undergoing apoptosis and decrease the number of cancer cells progressing through the cell cycle, these benefits are not consistent for all the concentrations examined. These results are consistent with the results of the MCF-7 cell proliferation assays, and show that these compounds have an estrogenic effect at some concentrations, but act as an anti-estrogen at other concentrations. This suggests that there may be an optimal concentration at which LY5 and LY8 may exert full estrogen-like activity, whereas at other concentrations it may have partial antiestrogenic effects. This characteristic of compounds LY5 and LY8 limits their potential use as a therapeutic agent since they appear to have the potential to function both as an estrogen and an anti-estrogen on breast epithelial cells depending on the concentration. A similar differential effect of low or

high dosages was reported by Simoncini et al (25) with the experimental SERM aclobifene on eNOS activity in aortas of estrogen deficient rats, as noted in the Results section.

Nitric oxide (NO) synthesized by endothelial cells is a potent anti-inflammatory and antiatherogenic factor that is able to prevent endothelial cell dysfunction. Furthermore, NO is of principal importance for the regulation of vascular tone, and for the control of hemostasis (194). Estrogen stimulates the synthesis and release of NO through the regulation of both transcriptional and non transcriptional eNOS activity (25,189,195,196). Rapid NO synthesis upon exposure to estrogen relies on nongenomic activation of MAPK and PI3K/Akt pathways (25,195,196). For this reason, we evaluated the effects of the selected compounds on the rapid induction of Akt and eNOS phosphorylation, and hence the rapid production of NO. The optimal SERM would be expected to stimulate the rapid synthesis and release of NO at levels similar to estrogen. Unfortunately, the compounds tested did not induce phosphorylation of e-NOS at levels observed with stimulation by estrogen. It should be noted, however, that the LY doses used to test for induction of NO synthesis were chosen based on the results from the cell proliferation assays and the cell cycle analysis experiments. It is possible that these compounds may exhibit a more favorable profile in terms of NO induction at concentrations other than the ones examined in theses experiments.

According to the results of this study, LY11 appears to have the most favorable profile of the compounds examined. LY 11 treatment results in increased endothelial cell proliferation for concentrations lower or equal to $10^{-6}$M. LY11 exerts concentration

dependent growth inhibition of MCF-7 breast cancer cells with an $IC_{50}$-value of approximately $10^{-6}$M, close to the $IC_{50}$-value of $0.9x10^{-6}$M of tamoxifen (199). These findings are also consistent with the results of the cell cycle analysis for MCF-7 cells that showed LY11 causeds a significant decrease in survival of breast cancer cells, as evidenced by the increase in apoptosis. It also causes a significant decrease in the progression of the cell cycle to the S and G2/M phases, as compared to estrogen. LY11 also appears to antagonize the survival benefit conferred by estrogen on the breast cancer cells, as evidenced by the increase in apoptosis when the two treatment are combined.

In summary, the present study has demonstrated a potentially beneficial vascular effect of a novel SERM, while acting as an estrogen antagonist and an inhibitor of growth and survival in the breast cancer cell line MCF-7. As the molecular mechanisms of the action of SERMs become more completely understood, rational drug design will replace the current empirical method for the discovery of new SERMs. It is very likely that the ultimate goal of SERM research will be achieved, that is, the discovery of a tissue-selective drug that has all the beneficial effects of estrogen, including superagonist protective actions on the cardiovascular and skeletal systems, has none of its adverse effects, and offers protection against breast cancer. Even more exciting may be the possibility that the plasticity of estrogen action exhibited by SERMs will also be found to be a general feature of the steroid nuclear-receptor family of molecules.

**References**


1. J. Stamler, R. Pick and L.N. Katz , Prevention of coronary atherosclerosis by estrogen-androgen administration in the cholesterol fed chick. *Circ Res* **1** (1953), p. 94.
2. D.R. Harder and P.B. Coulson , Estrogen receptors and effects of estrogen on membrane electrical properties of coronary vascular smooth muscle. *J Cell Physiol* **100** (1979), pp. 375–382.
3. J.K. Williams, M.R. Adams and H.S. Klopfenstein , Estrogen modulates responses of atherosclerotic coronary arteries. *Circulation* **81** (1990), pp. 1680–1687.
4. J.K. Williams, M.R. Adams, D.M. Herrington and T.B. Clarkson , Short-term administration of estrogen and vascular responses of atherosclerotic coronary arteries. *J Am Coll Cardiol* **20** (1992), pp. 452–457.
5. Rubanyi, G.M. and Ryan, U.S., Editors, 1991. *Role of Endothelium in Regulation of Vascular Tone*, Marcel Dekker, New York.
6. Maddox, Y.T., Falcon, J.G., Ridinger, M., Cunard, C.M. and Ramwell, P.W., 1987. Endothelium dependent gender differences in the response of the rat aorta. *J. Pharmacol. Exp. Ther.* **240**, pp. 392–395.
7. Stallone, J.N., Crofton, J.T. and Share, L., 1991. Sexual dimorphism in vasopressin-induced contraction of rat aorta. *Am. J. Physiol.* **260**, pp. H453–H458.
8. Hayashi, T., Fukuto, J.M., Ignarro, L.J. and Chaudhuri, G., 1995. Gender differences in atherosclerosis: possible role of nitric oxide. *J. Cardiovasc. Pharmacol.* **26**, pp. 792–802.
9. Kauser, K. and Rubanyi, G.M., 1994. Gender difference in bioassayable endothelium-derived nitric oxide release from isolated rat aortae. *Am. J. Physiol.* **267**, pp. H2311–H2317.
10. Gisclard, V., Miller, V.N. and Vanhoutte, P.M., 1988. Effect of 17β-estradiol on endothelium dependent responses in the rabbit. *J. Pharmacol. Exp. Ther.* **244**, pp. 19–22.
11. Kauser, K. and Rubanyi, G.M., 1995. Effect of 17β-estradiol on endothelial dysfunction in the aorta of spontaneously hypertensive rats. *Hypertension* **25**, pp. 517–523.
12. Reis, S.E., Gloth, S.T., Blumenthal, R.S., Resar, J.R., Zacur, H.A., Gerstenblith, G. and Brinker, J.A., 1994. Ethinyl estradiol acutely attenuates abnormal coronary vasomotor responses to acetylcholine in postmenopausal women. *Circulation* **89**, pp. 52–60.
13. Rosselli, M., Imthurn, B., Keller, P.J., Jackson, E.K. and Dubey, R.K., 1995. Circulating nitric oxide (nitrite/nitrate) levels in postmenopausal women substituted with 17β-estradiol and norethisterone acetate. *Hypertension* **25**, pp. 848–853.
14. V. Lindner, S.K. Kim, R.H. Karas, G.G. Kuiper, J.A. Gustafsson and M.E. Mendelsohn , Increased expression of estrogen receptor-beta mRNA in male blood vessels after vascular injury. *Circ Res* **83** (1998), pp. 224–229.

15. R.H. Karas, B.L. Patterson and M.E. Mendelsohn , Human vascular smooth muscle cells contain functional estrogen receptor. *Circulation* **89** (1994), pp. 1943–1950.
16. M.E. Mendelsohn and R.H. Karas , Estrogen and the blood vessel wall. *Curr Opin Cardiol* **9** (1994), pp. 619–626.
17. S.M. Cowley, S. Hoare, S. Mosselman and M.G. Parker , Estrogen receptors alpha and beta form heterodimers on DNA. *J Biol Chem* **272** (1997), pp. 19858–19862.
18. H. Shibata, T.E. Spencer, S.A. Onate, G. Jenster, S.Y. Tsai, M.J. Tsai and B.W. O'Malley , Role of co-activators and co-repressors in the mechanism of steroid/thyroid receptor action. *Recent Prog Horm Res* **52** (1997), pp. 141–164.
19. M.E. Mendelsohn and R.H. Karas , The protective effects of estrogen on the cardiovascular system. *N Engl J Med* **340** (1999), pp. 1801–1811.
20. M.E. Mendelsohn, Genomic and Nongenomic Effects of Estrogen in the Vasculature. *Am J Cardiol* **90** (2002), (suppl):3F-6F
21. T. Caulin-Glaser, G. García-Cardeña, P. Sarrel, W.C. Sessa and J.R. Bender , 17 beta-estradiol regulation of human endothelial cell basal nitric oxide release, independent of cytosolic $Ca^{2+}$ mobilization. *Circ Res* **81** (1997), pp. 885–892.
22. R.L. Lantin-Hermoso, C.R. Rosenfeld, I.S. Yuhanna, Z. German, Z. Chen and P.W. Shaul , Estrogen acutely stimulates nitric oxide synthase activity in fetal pulmonary artery endothelium. *Am J Physiol* **273** (1997), pp. L119–L126.
23. Z. Chen, I.S. Yuhanna, Z. Galcheva-Gargova, R.H. Karas, M.E. Mendelsohn and P.W. Shaul , Estrogen receptor α mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. *J Clin Invest* **103** (1999), pp. 401–406.
24. M.P. Haynes, D. Sinha, K.S. Russell, M. Collinge, D. Fulton, M. Morales-Ruiz, W.C. Sessa and J.R. Bender , Membrane estrogen receptor engagement activates endothelial nitric oxide synthase via the PI3-kinase-Akt pathway in human endothelial cells. *Circ Res* **87** (2000), pp. 677–682.
25. T. Simoncini, A. Hafezi-Moghadam, D.P. Brazil, K. Ley, W.W. Chin and J.K. Liao , Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* **407** (2000), pp. 538–541.
26. K.L. Chambliss, I.S. Yuhanna, C. Mineo, P. Liu, Z. German, T.S. Sherman, M.E. Mendelsohn, R.G.W. Anderson and P.W. Shaul , Estrogen receptor α and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae. *Circ Res* **87** (2000), pp. e44–e52.
27. Rubanyi, G.M., Editor, , 1992. *Endothelin*, Oxford University Press, New York.
28. Busse, R. and Fleming, I., 1996. Endothelial dysfunction in atherosclerosis. *J. Vasc. Res.* **33**, pp. 181–194.
29. Williams, J.K., Adams, M.R. and Klopfenstein, H.S., 1990. Estrogen modulates responses of atherosclerotic coronary arteries. *Circulation* **81**, pp. 1680–1687.
30. Cayatte, A.J., Palacino, J.J., Horten, K. and Cohen, R.A., 1994. Chronic inhibition of nitric oxide production accelerates neointima formation and impairs endothelial function in hypercholesterolemic rabbits. *Arterioscler. Thromb.* **14**, pp. 753–759.
31. Naruse, K.M., Shimizu, M., Muramatsu, M., Toki, Y., Miyazaki, Y., Okurama, K., Hashimoto, H. and Ito, T., 1994. Long-term inhibition of NO synthesis promotes

atherosclerosis in the hypercholesterolemic rabbit thoracic aorta. *Arterioscler. Thromb.* **14**, pp. 746–752.

32. Kauser, K., daCunha, V., Fitch, R., Mallari, C. and Rubanyi, G.M., 2000. Role of endogenous nitric oxide in the progression of atherosclerosis in apolipoprotein E-deficient mice. *Am. J. Physiol.* **278**, pp. H1679–H1685.
33. Nascimento, C.A., Kauser, K. and Rubanyi, G.M., 1999. Effect of 17beta-estradiol in hypercholesterolemic rabbits with severe endothelial dysfunction. *Am. J. Physiol.* **276**, pp. H1788–H1794.
34. Holm, P., Korsgaard, N., Shalmi, M., Andersen, H.L., Hougaard, P. and Skouby, S.O., 1997. Significant reduction of the antiatherogenic effect of estrogen by long-term inhibition of nitric oxide synthesis in cholesterol-clamped rabbits. *J. Clin. Invest.* **100**, pp. 821–828.
35. Winber, N., Hoegholm, A., Christensen, H.R., Bang, L.E., Mikkelsen, K.L., Nielsen, P.E., Svendsen, T.L., Kampmann, J.P., Madsen, N.H. and Bentzon, M.W., 1995. 24-h Ambulatory blood pressure in 352 normal Danish subjects, related to age and gender. *Am. J. Hypertens.* **8**, pp. 978–986.
36. Ganten, U., Schroder, G., Witt, M., Zimmerman, F., Ganten, D. and Stock, G., 1989. Sexual dimorphism of blood pressure in spontaneously hypertensive rats: effects of anti-androgen treatment. *J. Hypertens.* **7**, pp. 721–726.
37. Rowland, N.E. and Fregly, M.J., 1992. Role of gonadal hormones in hypertension in the Dahl salt-sensitive rat. *Clin. Exp. Hypertens.* **A14**, pp. 367–375.
38. Ouchi, Y., Share, L., Crofton, J.T., Iitake, K. and Brooks, D.P., 1987. Sex difference in the development of deoxycorticosterone-salt hypertension in the rat. *Hypertension* **9**, pp. 172–177.
39. Burl, V.L., Whelton, P., Roccella, E.J., Brown, C., Cutler, J.A., Higgins, M., Horan, M.J. and Labarthe, D., 1995. Prevalance of hypertension in the US adult population: results from the Third National Health and Nutrition Examination Survey, 1988–1991. *Hypertension* **25**, pp. 305–313.
40. Hinojosa-Laborde, C., Lange, D.L. and Haywood, J.R., 2000. Role of female sex hormones in the development and reversal of Dahl hypertension. *Hypertension* **35**, pp. 484–489.
41. Seely, E.W., Walsh, B.W., Gerhard, M.D. and Williams, G.W., 1999. Estradiol with or without progesterone and ambulatory blood pressure in postmenopausal women. *Hypertension* **33**, pp. 1190–1194.
42. Kauser, K. and Rubanyi, G.M., 1997. Potential cellular signaling mechanisms mediating upregulation of endothelial nitric oxide production by estrogen. *J. Vasc. Res.* **34**, pp. 229–236.
43. Kauser, K. and Rubanyi, G.M., 1998. 17β-Estradiol augments endothelial nitric oxide production in the aortae of male sponatenously hypertensive rats. In: Moncada, S., Feelisch, M., Busse, R. and Higgs, E.A., Editors, 1998. *The Biology of Nitric Oxide*, Springer, Germany, pp. 13–18.
44. Reis, S.E., Gloth, S.T., Blumenthal, R.S., Resar, J.R., Zacur, H.A., Gerstenblith, G. and Brinker, J.A., 1994. Ethinyl estradiol acutely attenuates abnormal coronary vasomotor responses to acetylcholine in postmenopausal women. *Circulation* **89**, pp. 52–60.

45. Rosselli, M., Imthurn, B., Keller, P.J., Jackson, E.K. and Dubey, R.K., 1995. Circulating nitric oxide (nitrite/nitrate) levels in postmenopausal women substituted with 17β-estradiol and norethisterone acetate. *Hypertension* **25**, pp. 848–853.
46. Koos, R.D. and Le Maire, W.J., 1983. Factors that may regulate the growth and regression of blood vessels in the ovary. *Semin. Reprod. Endocrinol.* **1**, pp. 295–307.
47. Modlich, U., Kaup, F.-J. and Augustin, H.G., 1996. Cyclic angiogenesis and blood vessel regression in the ovary: blood vessel regression during luteolysis involves endothelial detachment and vessel occlusion. *Lab. Invest.* **74**, pp. 771–780.
48. Findlay, J.K., 1986. Angiogenesis in reproductive tissues. *J. Endocrinol.* **111**, pp. 357–366.
49. Reynolds, L.P., Killielea, S.D. and Redmer, D.A., 1992. Angiogenesis in the female reproductive system. *FASEB J.* **6**, pp. 886–892.
50. Baird, D.T., 2000. Overview of advances in contraception. *Br. Med. Bull.* **56**, pp. 704–716.
51. Rodger, F.R., Young, F.M., Fraser, H.M. and Illingworth, P.J., 1997. Endothelial cell proliferation follows the mid-cycle luteinizing hormone surge, but not human chorionic gonadotropin rescue, in the human corpus luteum. *Hum. Reprod.* **12**, pp. 1723–1729.
52. Naftolin, F. and Tolis, G., 1978. Neuroendocrine regulation of the menstrual cycle. *Clin. Obstet. Gynecol.* **21**, pp. 17–29.
53. Meyer, G.T. and McGeachie, J.K., 1988. Angiogenesis in the developing corpus luteum of pregnant rats: a stereologic and autoradiographic study. *Anat. Rec.* **222**, pp. 18–25.
54. Kaiserman-Abramof, I.R. and Padykula, H.A., 1989. Angiogenesis in the postovulatory primate endometrium: the coiled arteriolar system. *Anat. Rec.* **224**, pp. 479–489.
55. Rissau, W. and Flamme, I., 1995. Vasculargenesis. *Annu. Rev. Cell Dev. Biol.* **11**, pp. 73–91.
56. Morales, D.E., McGowan, K.A., Grant, D.S., Maheshwari, S., Bhartiya, D., Cid, M.C., Kleinman, H.K. and Schnaper, H.W., 1995. Estrogen promotes angiogenic activity in human umbilical vein endothelial cells in vitro and in a murine model. *Circulation* **91**, pp. 755–763.
57. Kleinman, H.K., Malinda, K.M., Gho, Y.S. and Ponce, M.L., 2000. In vitro and in vivo models of angiogenesis. In: Rubanyi, G.M., Editor, , 2000. *Angiogenesis in Health and Disease*, Marcel Dekker, New York, pp. 291–300.
58. Kyriadides, Z.S., Petinakis, P., Kaklamanis, L., Sbarouni, E., Karayannakos, P., Iliopoulos, D., Dontas, I. and Kremastinos, D.T., 2001. Intramuscular administration of estrogen may promote angiogenesis and perfusion in rabbit model of chronic limb ischemia. *Cardiovasc. Res.* **49**, pp.
59. Shifren, J.L., Tseng, J.F., Zaloudek, C.J., Ryan, I.P., Meng, Y.G., Ferrara, N., Jaffe, R.B. and Taylor, R.N., 1996. Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for

angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis. *J. Clin. Endocrinol. Metab.* **81**, pp. 3112–3118.

60. Greb, R.R., Heikinheimo, O., Williams, R.F., Hodgen, G.D. and Goodman, A.L., 1997. Vascular endothelial growth factor in primate endometrium is regulated by oestrogen-receptor and progesterone-receptor ligands in vivo. *Hum. Reprod.* **12**, pp. 1280–1292.
61. Krasinski, K., Spyridopoulos, I., Asahara, T., van der Zee, R., Isner, J.M. and Losordo, D.W., 1997. Estradiol accelerates functional endothelial recovery after arterial injury. *Circulation* **95**, pp. 1768–1772.
62. Ferrara, N., 2000. The role of vascular endothelial growth factor in angiogenesis. In: Rubanyi, G.M., Editor, , 2000. *Angiogenesis in Health and Disease*, Marcel Dekker, New York, pp. 44–74.
63. Hyder, S.M., Stancel, G.M., Ghiappetta, C., Murthy, L., Boettger-Tong, H.L. and Makela, S., 1996. Uterine expression of vascular endothelial growth factor is increased by estradiol and tamoxifen. *Cancer Res.* **56**, pp. 3954–3960.
64. Rusnati, M., Casaroti, G., Pecorelli, S., Ragnotti, G. and Presta, M., 1993. Estro-progestinic replacement therapy modulates the levels of basic fibroblast growth factor (bFGF) in postmenopausal endometrium. *Gynecol. Oncol.* **48**, pp. 88–93.
65. Johns, A. and Zollner, S., 2000. Nitric oxide and angiogenesis. In: Rubanyi, G.M., Editor, , 2000. *Angiogenesis in Health and Disease*, Marcel Dekker, New York, pp. 191–198.
66. Cid, M.C., Kleinman, H.K., Grant, D.S., Schnaper, H.W., Fauci, A.S. and Hoffman, G.S., 1994. Estradiol enhances leukocyte binding to tumor necrosis factor (TNF)-stimulated endothelial cells via an increase in TNF-induced adhesion molecules E-selectin, intercellular adhesion molecule type 1, and vascular cell adhesion molecule type 1. *J. Cell Sci.* **93**, pp. 17–25.
67. Grant, D.S., Tashiro, K., Segui-Real, B., Yamada, Y., Martin, G.R. and Kleinman, H.K., 1989. Two different domains of laminin mediate the differentiation of human endothelial cells into capillary-like structures in vitro. *Cell* **58**, pp. 933–943.
68. Schecter, J. and Weiner, R., 1991. Changes in basic fibroblast growth factor coincident with estradiol-induced hyperplasia of the anterior pituitaries of Fisher 344 and Sprague–Dawley rats. *Endocrinology* **129**, pp. 2400–2408.
69. Spyridopoulos, I., Sullivan, A.B., Kearbey, M., Isner, J.M. and Losordo, D.W., 1997. Estrogen receptor-mediated inhibition of human endothelial cell apoptosis: estradiol as a survival factor. *J. Clin. Invest.* **98**, pp. 426–433.
70. Alvarez, R.J., Gips, S.J., Moldovan, N., Wilhide, C.C., Milliken, E.E., Hoang, A.T., Hruban, R.H., Silverman, H.S., Dang, C.V. and Goldschmidt-Clermont, P.J., 1997. 17β-Estradiol inhibits apoptosis of endothelial cells. *Biochem. Biophys. Res. Commun.* **237**, pp. 372–381.
71. Pike MC, Spicer DV, Dahmoush L, Press MF. Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk. Epidemiol Rev. 1993;**15**:17–35.
72. Endogenous Hormones and Breast Cancer Collaborative Group. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J Natl Cancer Inst. 2002;**94**:606–616.

73. Cuzick J, Powles T, Veronesi U, Forbes J, Edwards R, Ashley S, Boyle P. Overview of the main outcomes in breast-cancer prevention trials. Lancet. 2003;**361**:296–300.
74. Collaborative Group on Hormonal Factors in Breast Cancer. Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Lancet 1997;350:1047–59.
75. Schairer C, Lubin J, Troisi R, Sturgeon S, Brinton L, Hoover R. Menopausal estrogen and estrogen–progestin replacement therapy and breast cancer risk. JAMA 2000;283:485–91.
76. Clevenger CV, Furth PA, Hankinson SE, Schuler LA. The role of prolactin in mammary carcinoma. Endocr Rev. 2003;**24**:1–27.
77. Lillie EO, Bernstein L, Ursin G. The role of androgens and polymorphisms in the androgen receptor in the epidemiology of breast cancer. Breast Cancer Res. 2003;**5**:164–173.
78. McManus MJ, Welsch CW. The effect of estrogen, progesterone, thyroxine, and human placental lactogen on DNA synthesis of human breast ductal epithelium maintained in athymic nude mice. Cancer. 1984;**54**:1920–1927.
79. Cohen SM, Ellwein LB. Cell proliferation in carcinogenesis. Science. 1990;**249**:1007–1011.
80. Henderson BE, Feigelson HS. Hormonal carcinogenesis. Carcinogenesis. 2000;**21**:427–433.
81. Clemons M, Goss P. Estrogen and the risk of breast cancer. N Engl J Med. 2001;**344**:276–285.
82. Trichopoulos D. Hypothesis: does breast cancer originate *in utero*? Lancet. 1990;**335**:939–940.
83. Hilakivi-Clarke L, Cho E, Raygada M, Kenney N. Alterations in mammary gland development following neonatal exposure to estradiol, transforming growth factor alpha, and estrogen receptor antagonist ICI 182,780. J Cell Physiol. 1997;**170**:279–289. [
84. Russo IH, Russo J. Role of hormones in mammary cancer initiation and progression. J Mammary Gland Biol Neoplasia. 1998;**3**:49–61.
85. McCormack VA, dos Santos Silva I, De Stavola BL, Mohsen R, Leon DA, Lithell HO. Fetal growth and subsequent risk of breast cancer: results from long term follow up of Swedish cohort. BMJ. 2003;**326**:248–251.
86. Welty FK. Who should receive hormone replacement therapy? *J Thromb Thrombol*. 1996;3:13–21.
87. Campos H, McNamara JR, Wilson PWF, et al. Differences in low density lipoprotein subfractions and apolipoproteins in premenopausal and postmenopausal women. *J Clin Endocrinol Metab*. 1988;67:30–35.
88. Stevenson JC, Crook D, Godsland IF. Influence of age and menopause on serum lipids and lipoproteins in healthy women. *Atherosclerosis*. 1993;98:83–90.
89. Manolio TA, Pearson TA, Wenger NK, et al. Cholesterol and heart disease in older persons and women: review of an NHLBI workshop. *Ann Epidemiol*. 1992;2:161–176.

90. Walsh JM, Grady D. Treatment of hyperlipidemia in women. *JAMA*. 1995;274:1152–1158.
91. Kannel WB, Wilson PW. Risk factors that attenuate the female coronary risk advantage. *Arch Intern Med*. 1995;155:57–61.
92. Rath M, Niendorf A, Reblin T, et al. Detection and quantification of lipoprotein (a) in the arterial wall of 107 coronary bypass patients. *Arteriosclerosis*. 1989;9:570–592.
93. Maher VMG, Brown BG, Marcovina SM, et al. Effects of lowering elevated LDL cholesterol on the cardiovascular risk of lipoprotein(a). *JAMA*. 1995;274:1771–1774.
94. Niendorf A, Rath M, Wolf K, et al. Morphologic detection and quantification of lipoprotein (a) deposition in atheromatous lesions of human aorta and coronary arteries. *Virchows Arch A Pathol Anat*. 1990;417:105–111.
95. Bostom AG, Gagnon DR, Cupples A, et al. A prospective investigation of elevated lipoprotein (a) detected by electrophoresis and cardiovascular disease in women: the Framingham Heart Study. *Circulation*. 1994;90:1688–1695.
96. Colditz GA, Willett WC, Stampher MJ, et al. Menopause and the risk of coronary heart disease in women. *N Engl J Med*. 1987;316:1105–1110.
97. Writing Group for the Women's Health Initiative. Risks and benefits of estrogen plus progestin in healthy postmenopausal women. Principal results from the Women's Health Initiative randomized controlled trial. *JAMA*. 2002;288:321–333.
98. Hulley S, Grady D, Bush T, et al. Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. *JAMA*. 1998;280:605–613.
99. Mosca L, Collins P, Herrington DM, et al. Hormone replacement therapy and cardiovascular disease: a statement for healthcare professionals for the American Heart Association. *Circulation*. 2001;104:499–503.
100. Laine C. Postmenopausal hormone replacement therapy: how could we have been so wrong? *Ann Intern Med*. 2002;137:290.
101. Miller AP, Oparil S. Secondary prevention of coronary heart disease in women: a call to action. *Ann Intern Med*. 2003;138:150–151.
102. Herrington DM. Hormone replacement therapy and heart disease. Replacing dogma with data. *Circulation*. 2003;107:2–4.
103. Grodstein F, Clarkson TB, Manson JE. Understanding the divergent data on postmenopausal hormone therapy. *N Engl J Med*. 2003;348:645–650.
104. Grodstein F, Stampher M. The epidemiology of coronary heart disease and estrogen replacement in postmenopausal women. *Prog Cardiovasc Dis*. 1995;38:199–210.
105. Stampfer MJ, Coldtiz GA. Estrogen replacement therapy and coronary heart disease: a quantitative assessment of the epidemiological evidence. *Prev Med*. 1991;20:47–63.
106. Stampfer MH, Willet WC, Colditz GA, et al. A prospective study of postmenopausal estrogen therapy coronary heart disease. *N Engl J Med*. 1985;313:1044–1049.

107. Knopp RH, Zhu X, Bonet B. Effect of estrogens on lipoprotein metabolism and cardiovascular disease in women. *Atherosclerosis.* 1994:S83–S91.

108. The Writing Group for the PEPI Trial. Effects of estrogen/progestin regimens on heart disease in postmenopausal women. The Postmenopausal Estrogen/Progestin Interventions (PEPI) trial. *JAMA.* 1995;273:199–208.

109. Herrington DM, Howard TD, Hawkins GA, et al. Estrogen receptor polymorphisms and estrogen replacement on high-density lipoprotein cholesterol in women with coronary disease. *N Engl J Med.* 2002;346:967–974.

110. Massafra C, Buonocore G, Gioia D, et al. Effects of estradiol and medroxyprogesterone–acetate treatment on erythrocyte antioxidant enzyme activities and malondialdehyde plasma levels in amenorrheic women. *J Clin Endocrinol Metab.* 1997;82:173–175.

111. Bar J, Tepper R, Fuchs J, et al. The effect of estrogen replacement therapy on platelet aggregation and adenosine triphosphate release in postmenopausal women. *Obstet Gynecol.* 1993;81:261–264.

112. Williams AK, Adams MR, Klopfenstein HS. Estrogen modulates responses of atherosclerotic coronary arteries. *Circulation.* 1990;81:1680–1687.

113. Skafar DF, Rui XU, Morales J, et al. Clinical review 91: female sex hormones and cardiovascular disease in women. *J Clin Endocrinol Metab.* 1997;82:3913–3918.

114. Holm P, Korsgaard N, Shalmi M, et al. Significant reduction of the antiatherogenic effect of estrogen by long-term inhibition of nitric oxide synthesis in cholesterol-clamped rabbits. *J Clin Invest.* 1997;100:821–828.

115. Chen SJ, Li H, Durand J, et al. Estrogen reduces myointimal proliferation after balloon injury of rat carotid artery. *Circulation.* 1996;1993:577–584.

116. Chen SJ, Li H, Durand J, et al. Estrogen reduces myointimal proliferation after balloon injury of rat carotid artery. *Circulation.* 1996;1993:577–584.

117. Nabulsi AA, Folsom AR, White A, et al. Association of hormone-replacement therapy with various cardiovascular risk factors in women. Atherosclerosis Risk in Communities Study investigators. *N Engl J Med.* 1993;328:1069–1075.

118. Koh KK, Mincemoyer R, Bui MN, et al. Effects of hormone replacement therapy on fibrinolysis in postmenopausal women. *N Engl J Med.* 1997;336:683–690.

119. Salomaa V, Rasi V, Kekkanen J, et al. Association of hormone replacement therapy with hemostatic and other cardiovascular risk factors: the FINRISK hemostasis study. *Arterioscler Thromb Vasc Biol.* 1995;15:1549–1555.

120. Sullivan JM, Vander Zwaag R, Hughes JP, et al. Estrogen replacement and coronary artery disease: effect on survival in postmenopausal women. *Arch Intern Med.* 1990;150:2557–2562.

121. Nabulsi AA, Folsom AR, Szklo M, et al. No association of menopause and hormone replacement therapy with carotid artery intima–media thickness. *Circulation.* 1996;94:1857–1863.

122. Hodis NH, Mack WJ, Lobo RA, et al. Estrogen in the prevention of atherosclerosis. *Ann Intern Med.* 2001;135:939–953.

123. Stampfer MJ, Colditz GA, Willett WC, et al. Postmenopausal estrogen therapy and cardiovascular disease: 10 year follow-up from the Nurses' Health Study. *N Engl J Med.* 1991;325:756–762.

124. Kriz–Silverstein D, Barrett–Connor E. Long-term postmenopausal hormone use, obesity, and fat distribution in older women. *JAMA.* 1996;275:46–49.

125. Haarbo J, Marslew U, Gotfredsen A, et al. Postmenopausal hormone replacement therapy prevents central distribution of body fat after menopause. *Metabolism.* 1991;40:1323–1326.

126. Lobo RA, Pickar JH, Wild RA, et al. Metabolic impact of adding medroxyprogesterone acetate to conjugated estrogen therapy in postmenopausal women. *Obstet Gynecol.* 1994;84:987–995.

127. Andersson B, Mattsson LA, Hahn L, et al. Estrogen replacement therapy decreases hyperandrogenicity and improves glucose homeostasis and plasma lipids in postmenopausal women with noninsulin-dependent diabetes mellitus. *J Clin Endocrinol Metab.* 1997;82:638–643.

128. Godsland IF, Crook D, Simpson R, et al. The effects of different formulations of oral contraceptive agents on lipid and carbohydrate metabolism. *N Engl J Med.* 1990;323:1375–1381.

129. Collaborative group on hormonal factors in breast cancer. Breast cancer and hormone replacement therapy: collaborative re-analysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. *Lancet.* 1997;350:1047–1059.

130. Schulman ST, Thiemann DR, Ouyang P, et al. Effects of acute hormone therapy on recurrent ischemia in postmenopausal women with unstable angina. *J Am Coll Cardiol.* 2002;39:231–237.

131. Dixon JM. Hormone replacement therapy and the breast. *BMJ.* 2001;323:1381–1382.

132. Hulley S, Furberg C, Barrett–Connor E, et al. Noncardiovascular disease outcomes during 6.8 years of hormone therapy. Heart and Estrogen/Progestin Replacement Study Follow-up (HERS-II). *JAMA.* 2002;22:58–66.

133. Lacey JV, Mink PJ, Lubin JH, et al. Menopausal hormone replacement therapy and risk of ovarian cancer. *JAMA.* 2002;288:334–341.

134. Noller KL. Estrogen replacement therapy and risk of ovarian cancer. *JAMA.* 2002;288:368–369.

135. Henderson VW, Paganini–Hill A, Miller BL, et al. Estrogen for Alzheimer's disease in women: randomized, double-blind, placebo-controlled trial. *Neurology.* 2000;54:295–301.

136. Mulnard RA, Cotman CW, Kawas C, et al. Estrogen replacement therapy for treatment of mild to moderate Alzheimer's disease: a randomized controlled trial. *JAMA* 200;283:1007–1015.

137. Pandya KJ, Raubertaus RF, Flynn PJ, et al. Oral clonidine in postmenopausal patients with breast cancer experiencing tamoxifen-induced hot flashes: a University of Rochester Cancer Center Community Clinical Oncology Program Study. *Ann Intern Med.* 2000;132:78–93.

138. Torgerson DJ, Bell–Syer SEM. Hormone replacement therapy and prevention of nonvertebral fractures: a meta-analysis of randomized trials. *JAMA*. 2001;285:2891–2897.

139. Lacassagne, A. 1936. Hormonal pathogenesis of adenocarcinoma of the breast. Am. J. Cancer **27:** 217-225

140. Jensen, E.V. & H.I. Jacobson. 1962. Basic guides to the mechanism of estrogen action. Recent Prog. Horm. Res. **18:** 387-414.

141. Jensen, E.V., G.E. Block, S. Smith *et al.* 1971. Estrogen receptors and breast cancer response to adrenalectomy. Natl. Cancer Inst. Monogr. **34:** 55-70.

142. Lerner, L.J. 1981. The first non-steroidal antiestrogen—MER 25. *In* Nonsteroidal Antioestrogens: Molecular Pharmacology and Antitumour Activity, pp. 1-6. Academic Press. Sydney.

143. Jordan, V.C. & T. Jaspan. 1976. Tamoxifen as an antitumour agent: oestrogen binding as a predictive test for tumour response. J. Endocrinol. **68:** 453-460.

144. Osborne, C.K., K. Hobbs & G.M. Clark. 1985. Effect of estrogens and antiestrogens on growth of human breast cancer cells in athymic nude mice. Cancer Res. **45:** 584-590.

145. Jordan, V.C., E. Phelps & J.U. Lindgren. 1987. Effects of anti-estrogens on bone in castrated and intact female rats. Breast Cancer Res. Treat. **10:** 31-35.

146. Turner, R.T., G.K. Wakley, K.S. Hannon *et al.* 1987. Tamoxifen prevents the skeletal effects of ovarian hormone deficiency in rats. J. Bone Miner. Res. **2:** 449-456.

147. Turner, R.T., G.K. Wakley, K.S. Hannon *et al.* 1988. Tamoxifen inhibits osteoclast-mediated resorption of trabecular bone in ovarian hormone-deficient rats. Endocrinology **122:** 1146-1150.

148. Jordan, V.C. & K.E. Allen. 1980. Evaluation of the antitumour activity of the non-steroidal antioestrogen monohydroxytamoxifen in the DMBA-induced rat mammary carcinoma model. Eur. J. Cancer **16:** 239-251

149. Harper, M.J. & A.L. Walpole. 1967. A new derivative of triphenylethylene: effect on implantation and mode of action in rats. J. Reprod. Fertil. **13:** 101-119.

150. Jordan, V.C. & S.P. Robinson. 1987. Species-specific pharmacology of antiestrogens: role of metabolism. Fed. Proc. **46:** 1870-1874.

151. Jordan, V.C., M.K. Lababidi & S. Langan-Fahey. 1991. Suppression of mouse mammary tumorigenesis by long-term tamoxifen therapy. J. Natl. Cancer Inst. **83:** 492-496

152. Gottardis, M.M., S.P. Robinson, P.G. Satyaswaroop *et al.* 1988. Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. Cancer Res. **48:** 812-815.

153. Love, R.R., R.B. Mazess, H.S. Barden *et al.* 1992. Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N. Engl. J. Med. **326:** 852-856

154. Love, R.R., D.A. Wiebe, P.A. Newcomb *et al.* 1991. Effects of tamoxifen on cardiovascular risk factors in postmenopausal women. Ann. Intern. Med. **115:** 860-864.

155. Powles, T.J., T. Hickish, J.A. Kanis *et al.* 1996. Effect of tamoxifen on bone mineral density measured by dual-energy X-ray absorptiometry in healthy premenopausal and postmenopausal women. J. Clin. Oncol. **14:** 78-84

156. Guetta, V., R.M. Lush, W.D. Figg, *et al.* 1995. Effects of the antiestrogen tamoxifen on low-density lipoprotein concentrations and oxidation in postmenopausal women. Am. J. Cardiol. **76:** 1072-1073.

157. Blum, A., W.H. Schenke, L. Hathaway, *et al.* 2000. Effects of estrogen and the selective estrogen receptor modulator raloxifene on markers of inflammation in postmenopausal women. Am. J. Cardiol. **86:** 892-895

158. Colditz, G.A., W.C. Willett, M.J. Stampfer, *et al.* 1987. Menopause and the risk of coronary heart disease in women. N. Engl. J. Med. **316:** 1105-1110.

159. Stampfer, M.J. & G.A. Colditz. 1991. Estrogen replacement and coronary heart disease: a quantitative assessment of the epidemiologic evidence. Prev. Med. **20:** 47-63.

160. Colditz, G.A., S.E. Hankinson, D.J. Hunter, *et al.* 1995. The use of estrogens and progestins and the risk of breast cancer in postmenopausal women. N. Engl. J. Med. **332:** 1589-1593.

161. Kuiper GGJM, Carlsson B, Grandien K, et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors $\alpha$ and $\beta$. Endocrinology 1997;138:863-870.

162. Couse JF, Korach KS. Estrogen receptor null mice: what have we learned and where will they lead us? Endocr Rev 1999;20:358-417.

163. Lindberg MK, Movérare S, Skrtic S, et al. Estrogen receptor-$\beta$ reduces estrogen receptor-$\alpha$ regulated gene transcription, supporting a "Ying Yang" relationship between estrogen receptor $\alpha$ and $\beta$ in mice. Mol Endocrinol (in press).

164. Pettersson K, Delaunay F, Gustafsson J-Å. Estrogen receptor $\beta$ acts as a dominant regulator of estrogen signaling. Oncogene 2000;19:4970-4978

165. Hall JM, McDonnell DP. The estrogen receptor $\beta$-isoform (ER$\beta$) of the human estrogen receptor modulates ER$\alpha$ transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology 1999;140:5566-5578.

166. Shiau AK, Barstad D, Loria PM, et al. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 1998;95:927-937.

167. Brzozowski AM, Pike AC, Dauter Z, et al. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 1997;389:753-758.

168. Paige LA, Christensen DJ, Grøn H, et al. Estrogen receptor (ER) modulators each induce distinct conformational changes in ER$\alpha$ and ER$\beta$. Proc Natl Acad Sci U S A 1999;96:3999-4004.

169. Pike ACW, Brzozowski AM, Walton J, et al. Structural insights into the mode of action of a pure antiestrogen. Structure (Camb) 2001;9:145-53.

170. McKenna NJ, Lanz RB, O'Malley BW. Nuclear receptor coregulators: cellular and molecular biology. Endocr Rev 1999;20:321-344.

171. Smith CL, Nawaz Z, O'Malley BW. Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. Mol Endocrinol 1997;11:657-666.

172. Norris JD, Paige LA, Christensen DJ, et al. Peptide antagonists of the human estrogen receptor. Science 1999;285:744-746.
173. Jepsen K, Hermanson O, Onami TM, et al. Combinatorial roles of the nuclear receptor corepressor in transcription and development. Cell 2000;102:753-763
174. Shang Y, Brown M. Molecular determinants for the tissue specificity of SERMs. Science 2002;295:2465-2468
175. Black, L.J., M. Sato, E.R. Rowley, *et al.* 1994. Raloxifene (LY139481 HCl) prevents bone loss and reduce serum cholesterol without causing uterine hypertrophy in ovariectomized rats. J. Clin. Invest. **93:** 63-69
176. Delmas, P.D., N.H. Bjarnason, B.H. Mitlak, *et al.* 1997. Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women. N. Engl. J. Med. **337:** 1641-1647
177. Walsh, B.W., L.H. Kuller, R.A. Wild, *et al.* 1998. Effects of raloxifene on serum lipids and coagulation factors in healthy postmenopausal women. JAMA **279:** 1445-1451.
178. Blum, A., L. Hathaway, R. Mincemoyer, *et al.* 2000. Hormonal, lipoprotein, and vascular effects of the selective estrogen receptor modulator raloxifene in hypercholesterolemic men. Am. J. Cardiol. **85:** 1491-1494
179. Bjarnason, N.H., J. Haarbo, I. Byrjalsen, *et al.* 1997. Raloxifene inhibits aortic accumulation of cholesterol in ovariectomized, cholesterol fed rabbits. Circulation **96:** 1964-1969.
180. Clarkson, T.B., M.S. Anthony & C.P. Jerome. 1998. Lack of effect of raloxifene on coronary artery atherosclerosis of postmenopausal monkeys. J. Clin. Endocrinol. Metab. **83:** 721-726.
181. Davies, M.J., J.L. Gordon, A.J.H. Gearing, *et al.* 1993. The expression of the adhesion molecules ICAM-1, VCAM-1, PECAM, and E-selectin in human atherosclerosis. J. Pathol. **171:** 223-229
182. Hwang, S.J., C.M. Ballantyne, R. Sharrett, *et al.* 1997. Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk in Communities (ARIC) study. Circulation **96:** 4219-4225
183. Ridker, P.M., C.H. Hennekens, B. Roitman-Johnson, *et al.* 1998. Plasma concentration of soluble intercellular adhesion molecule 1 and risks of future myocardial infarction in apparently healthy men. Lancet **351:** 88-92
184. Caulin-Glaser, T., W.J. Farrell, S.E. Pfau, *et al.* 1998. Modulation of circulating cellular adhesion molecules in postmenopausal women with coronary artery disease. J. Am. Coll. Cardiol. **31:** 1555-1560
185. Koh, K.K., C. Cardillo, M.N. Bui, *et al.* 1999. Vascular effects of estrogen and cholesterol-lowering therapies in hypercholesterolemic postmenopausal women. Circulation **99:** 354-360.
186. Blum, A., W.H. Schenke, L. Hathaway, *et al.* 2000. Effects of estrogen and the selective estrogen receptor modulator raloxifene on markers of inflammation in postmenopausal women. Am. J. Cardiol. **86:** 892-895

187. Li, L., Haynes, M.P., Bender, J.R.: Plasma Membrane Localization and Function of the Estrogen Receptor a Variant (ER46) in Human Endothelial Cells. Proc. Natl. Acad. Sci., USA., **100**:4807-4812, 2003

188. Russell, K. S. , Haynes, M. P. , Sinha, D. , Clerisme, E. & Bender, J. R. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 5930-5935

189. Haynes, M. P. , Sinha, D. , Russell, K. S. , Collinge, M. , Fulton, D. , Morales-Ruiz, M. , Sessa, W. C. & Bender, J. R. (2000) *Circ. Res.* **87**, 677-682

190. V. Craig Jordan, Susan Gapstur, and Monica Morrow (2001) J Natl Cancer Inst 2001; **93**: 1449-1457.

191. Simoncini T, Genazzani AR 2000 Direct vascular effects of estrogens and selective estrogen receptor modulators. Curr Opin Obstet Gynecol **12**:181–187

192. Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature.* 1993;**362**:801–809.

193. White CR, Shelton J, Chen SJ, et al. Estrogen restores endothelial cell function in an experimental model of vascular injury. *Circulation.* 1997;**96**:1624–1630.

194. Liao JK 1999 Endothelial nitric oxide and vascular inflammation. In: Panza JA, Cannon ROI, eds. Endothelium, nitric oxide and atherosclerosis. Armonk, NY: Futura; 119–132

195. Simoncini T, Genazzani AR 2000 Raloxifene acutely stimulates nitric oxide release from human endothelial cells via an activation of endothelial nitric oxide synthase. J Clin Endocrinol Metab **85**:2966–2969

196. Hisamoto K, Ohmichi M, Kurachi H, Hayakawa J, Kanda Y, Nishio Y, Adachi K, Tasaka K, Miyoshi E, Fujiwara N, Taniguchi N, Murata Y 2001 Estrogen induces the Akt-dependent activation of endothelial nitric-oxide synthase in vascular endothelial cells. J Biol Chem **276**:3459–3467

197. Prall, O. W. J., Rogan, E. M., and Sutherland, R. L. (1998) *J. Steroid Biochem. Mol. Biol.* **65**, 169-174

198. Nicholson, R. I., Gee, J. M., Bryant, S., Francis, A. B., McClelland, R. A., Knowlden, J., Wakeling, A. E., and Osborne, C. K. (1996) *Ann. N. Y. Acad. Sci.* **784**, 325-335

199. Larsen, S.S., Madsen, M.W., Jensen, B.L. and Lykkesfeldt, A.E., 1997. Resistance of human breast-cancer cells to the pure steroidal antiestrogen ICI 182,780 is not associated with a general loss of estrogen-receptor expression or lack of estrogen responsiveness. *Int. J. Cancer* **72**, pp. 1129–1136.

200. Edgell CJ, McDonald CC, Graham JB. Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc Natl Acad Sci U S A.* 1983;**80**:3734–3737.

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